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# 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 tumor 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)[1] 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 previously 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

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*The atomic coordinates and structure factors (code 1QR1) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).*

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[1] The abbreviations used are: MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; pMHC, peptide-MHC complex(es); APL, altered peptide ligand(s); HPLC, high pressure liquid chromatography; BFA, brefeldin A; A2, HLA-A2.1.

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 (IISAVVGIL), 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 $\beta_2$-microglobulin were expressed in *Escherichia coli*, produced as inclusion bodies, purified, and folded as described previously (17). Briefly, peptide, solubilized $\beta_2$-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 kept below 50 $\mu$g ml$^{-1}$. The solution was incubated at 10 °C for 36–48 h and then concentrated (Amicon) and purified by HPLC gel filtration (Phenomenex, BioSep-SEC-S2000).

*Synthetic Peptides*—All peptides were synthesized by the Peptide Synthesis Facility at the University of North Carolina at Chapel Hill. The peptides were purified to greater than 95% purity by reversed-phase HPLC and identity confirmed by matrix-assisted laser desorption ionization-time-of-flight spectroscopy. Peptides were dissolved in 100% dimethyl sulfoxide at 20 mg ml$^{-1}$ by weight. The final peptide 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 $KH_2/K_2HPO_4$ buffer, pH 7.5, and adjusted to a final protein concentration of 4–12 $\mu$M. The change in CD signal at 218 nm was measured as a function of temperature from 4 to 95 °C on an 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. $T_m$ 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, $2.5 \times 10^5$ T2 cells (ATCC CRL-1992) were incubated overnight in AIM V serum-free medium (Life Technologies, Inc.) at 37 °C, 5% $CO_2$ in the presence of GP2 or APL, at concentrations varying from 50 to 0.05 $\mu$M. Cells were then stained with the monoclonal antibody BB7.2 (18) specific for A2, followed by fluorescein isothiocyanate-labeled (1:50) goat anti-mouse IgG antibody (Southern Biotechnology Associates). Cells were analyzed by flow cytometry (FACScan, Becton Dickinson), and the mean channel fluorescence was determined using the CYCLOPS software package (Cytomation, Fort Collins, CO). All data are normalized as the percentage of the mean channel fluorescence for the calreticulin-signal-sequence peptide, ML, bound to A2 at 50 $\mu$M. Binding by the A2-specific antibody, BB7.2, was not dependent on the peptide bound because W6/32, an antibody that binds to an epitope between the $\alpha$3 domain and $\beta_2$-microglobulin, gave similar results (data not shown).

*Cell Surface Half-life Assay*—The determination of cell surface half-lives ($T_{1/2}$) of A2-peptide complexes was performed as described previously (11). Briefly, $2.5 \times 10^6$ T2 cells were incubated overnight in AIM V serum-free medium at 37 °C, 5% $CO_2$ in the presence of 50 $\mu$M peptide. To block the egress of new A2 molecules to the surface, cells were incubated at 37 °C, 5% $CO_2$ in RPMI 1640, 15% fetal calf serum and 10 $\mu$g 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 $\mu$g ml$^{-1}$ BFA. At the indicated time points, $2.5 \times 10^5$ 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.

TABLE I

*Summary of binding data of HER-2/neu-derived peptides to A2*

Residues substituted with respect to wild-type peptide are shown in boldface type. $T_m$ is the temperature (°C) at which 50% of the protein is denatured as measured by circular dichroism. $K_r$ is the relative binding constant as determined by the T2 cell surface assembly assay. $K_r$ is defined as the concentration of peptide in $\mu$M that yields 50% mean channel fluorescence as compared to the maximum fluorescence of the control peptide (ML) at 50 $\mu$M. The $K_r$ value for ML is the concentration that yields 50% mean channel fluorescence. $T_{1/2}$ is the half-life of peptide-A2 complexes (in hours) as determined by the T2 cell surface stability assay. ND, not determined. DNF, did not fold *in vitro*. The error in the $T_m$ is the sum of the machine and curve fit errors. It is typically about 1 °C. >50 means that the concentration to yield 50% of ML fluorescence is greater than 50 $\mu$M.

| Peptide | Sequence | $T_m$ | $K_r$ | $T_{1/2}$ |
|---|---|---|---|---|
| GP2 (16) | IISAVVGIL | 36.4 | >50 | 0.35 |
| S1 (38) | SIISAVVGI | 44.2 | 13.2 | 4.05 |
| L10 (38) | IISAVVGILL | 41.3 | >50 | 1.58 |
| E74 (15) | DVRLVHRDL | DNF | >50 | DNB |
| E75 (15) | KIFGSLAFL | 45.1 | 14.4 | 8.57 |
| F56 (39) | YISAWPDSL | 34.8 | >50 | DNB |
| C84 (39) | ELVSEFSRV | 37.5 | >50 | 0.30 |
| L9V | IISAVVGI**V** | 38.8 | >50 | 0.69 |
| I2L | I**L**SAVVGIL | 42.2 | 22.9 | 1.76 |
| I2L/L9V | I**L**SAVVGI**V** | 42.5 | 10.0 | 2.48 |
| ML (30) | MLLSVPLLL | 52.5 | 1.8 | 19.53 |
| RT (31) | ILKEPVHGV | 50.0 | 7.7 | 9.69 |
| MelA (36) | EAAGIGILTV | 40.9 | 47.2 | 0.44 |
| MelA-A2L | E**L**AGIGILTV | 50.0 | 1.6 | 9.98 |

*Crystallization, Data Collection and Processing*—Crystals were grown by hanging drop vapor diffusion as described previously (19). Crystallographic data of A2-GP2 were collected on a single crystal at the National Synchrotron Light Source, Brookhaven National Labs, beamline X-12B at −170 °C (Oxford Cryosystems). Evaluation of the diffraction pattern with DENZO autoindexing function showed the space group to be triclinic P1. 180 degrees of data were collected at a distance of 90 mm from the Quantum 4 CCD detector (ADSC Poway, CA) with one-degree oscillations and 3 min of exposure time/frame. Data were processed with DENZO, and intensities were scaled with SCALEPACK (20). Data statistics are shown in Table II.

*Structure Determination and Refinement*—The A2-GP2 structure was determined by molecular replacement using AMoRe (21) within the CCP4 program suite (22). The A2-hepatitis peptide complex (PDB accession code 1HHH) was used as the search model (23). Because the domains tend to vary in their relative orientations with respect to one another in different crystal forms, the search model was divided into three pieces, the peptide-binding superdomain ($\alpha_1\alpha_2$), the $\alpha_3$ domain, and $\beta_2$-microglobulin. Initial rounds of positional refinement used X-PLOR from 8–2.4 Å resolution data. Later rounds were performed with Refmac using all data from 30.0 to 2.4 Å. Final rounds of refinement used torsional dynamics with CNS (24–26) with all data. Electron density maps were generated using DM and functions for 2-fold non-crystallographic averaging, histogram matching, and solvent flattening. Manual intervention was performed using O (27). 103 water molecules were added to the structure using the program ARP (28) combined with Refmac and confirmed by visual inspection of the electron density maps. The refinement statistics are listed in Table II.

## 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 Bs were refined in the penultimate cycle followed by the addition of waters.

| | |
|---|---|
| **Data statistics** | |
| Space group | P1 |
| Cell Dimensions | $a$ = 50.34 Å<br>$b$ = 63.61 Å<br>$c$ = 75.14 Å<br>$\alpha$ = 81.98 °<br>$\beta$ = 76.25 °<br>$\gamma$ = 77.83 ° |
| Molecules/Asymmetric Unit | 2 |
| Resolution | 30–2.4 Å |
| $R_{merge}$ (%)[a] | 9.3 (23.3)[b] |
| $<I/\sigma>$ | 7.80 (3.46) |
| Unique reflections | 34,962 |
| Total reflections | 66,839 |
| Completeness (%) | 98.2 (97.6) |
| **Refinement** | |
| Resolution | 30–2.4 Å |
| $R_{free}$ (%) (number of reflections)[c] | 28.4 (1,714) |
| $R_{work}$ (%) (number of reflections)[c] | 24.2 (31,969) |
| *R*s fit[d] | 83.8% |
| No. of non-hydrogen atoms | 6,292 |
| No. of waters | 103 |
| Error[e] | 0.26 Å |
| Average B factor | 16.8 Å$^2$ |
| R.M.S. deviations from ideality | |
| Bonds | 0.009 Å |
| Angles | 1.468 ° |
| Residues in Ramachandran plot | |
| Most favored | 91.6% |
| Additional allowed | 8.1% |
| Generously allowed | 0.3% |
| Disallowed | 0.0% |

[a] $R_{merge} = \Sigma_{hkl} \Sigma_i |I_i - <I>| / \Sigma_{hkl} \Sigma_i I_i$, where $I_i$ is the observed intensity and $<I>$ is the average intensity of multiple observations of symmetry related reflections.

[b] Number in parenthesis refers to the highest resolution shell (2.44–2.40) for A2-GP2 unless otherwise stated.

[c] $R = \Sigma_{hkl} | |F_{obs}| - k |F_{cal}| | / \Sigma_{hkl} |F_{obs}|$, where $R_{free}$ is calculated for a randomly chosen 5% of reflections and $R_{work}$ is calculated for the remaining 95% of reflections used for structure refinement.

[d] *R*s fit is 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.

[e] Error is the mean estimate of the coordinate error based on maximum likelihood methods (Refmac).

circular dichroism, have been shown to correlate with the free energy of peptide binding to class I MHC (29). Therefore, the thermal stabilities of recombinant A2 complexes folded *in vitro* with seven HER-2/neu peptides identified as important epitopes for breast cancer immunotherapy in the literature (GP2, S1, L10, E74, E75, F56, and C84; see Table I for sequences) were determined. As can be seen from Fig. 1*A*, complexes formed with GP2, F56, and C84 have extremely low melting temperatures. Complexes formed with S1, L10, and E75 (summarized in Table I) have higher melting temperatures. E74 bound so poorly as to be undetectable in any of our assays (data not shown). A cell surface binding assay (Fig. 1*B*) using T2 cells with exogenously added peptide confirms the results found by the circular dichroism experiments. Two peptides, one hydrophobic and one hydrophilic, were chosen as representative "high affinity" binders. ML is derived from the signal sequence of calreticulin (30), and RT is derived from HIV-1 reverse transcriptase (31). The thermal stability ($T_m$) and the relative binding constant ($K_r$) determined by the T2 assay correlate well (91.3% correlation coefficient). This suggests that $K_r$ is proportional to $K_D$, because, as stated above, the $T_m$ has previously been shown to be proportional to the $K_D$ (29).

Adding BFA to the cell surface stability assay allows us to measure the amount of time a peptide-MHC complex stays on the surface of cells. As can be seen in Fig. 1*C*, the GP2 peptide has an extremely short half-life of ~21 min at 37 °C (Table I). Some of the other HER-2/neu-derived peptides have longer half-lives, but none are as long as peptides such as ML or RT (Fig. 1*C* and Table I). We could not detect binding of the E74 peptide in any of our assays. The fact that CTL activity toward E74 can be seen and we cannot measure binding is probably a function of the extreme sensitivity of CTL. Only ~100 class I-peptide complexes/cell are required to trigger an activated T cell (32).

*Anchor Substitutions Do Not Significantly Improve Binding of GP2*—Substitutions at peptide anchor positions have been shown to greatly increase the thermal stability of an influenza matrix peptide (12). In the present work, we chose one of the three poor binding peptides, GP2, to study the anchor substitutions of HER-2/neu-derived peptides. The GP2 anchors (Ile at position 2 and Leu at position 9 of the peptide) are found in peptides that bind with high affinity to A2, but these anchors are not optimal (8). Therefore, optimized APL based on GP2 were synthesized that replaced the Ile at position 2 with Leu (I2L) or the Leu at position 9 with Val (L9V). As can be seen in Fig. 1*D*, these substitutions did increase the thermal stability (~2–6 °C) but not to the degree that was seen for similar substitutions in the influenza matrix peptide (~7–9 °C) (12) or for a variant of a melanoma peptide (MelA and MelA-A2L, ~9 °C). The cell surface stability assay using T2 cells supports the CD data that we have measured (Fig. 1*E*). The half-lives of the APL complexes on the cell surface are increased with respect to GP2 (Fig. 1*F*). However, they are not close to the time constants seen for the positive control, high affinity binders ML or RT.

*Crystallographic Structure of A2-GP2*—To understand why GP2 binds poorly to A2 and why the anchor substitutions do not significantly increase the stability, we determined the crystallographic structure of A2-GP2. The molecular replacement solution was unambiguous with a correlation coefficient of ~73% after rigid body fitting. The model was refined in X-PLOR (33). During refinement, the peptide was omitted to reduce the potential for model bias. Density modification was performed with DM (22) using the X-PLOR output coordinates to generate unbiased averaged electron density maps of the peptide and to fit the structure of A2. Unlike all of the pMHC structures that we have determined to date, the entire length of the main chain of the peptide was not visible in the density modified electron density maps at this stage. After 10 cycles of model building with O (27) and computational refinement with X-PLOR and Refmac and finally with CNS, the refinement converged to the statistics shown in Table II. In general, the maps are clear and unambiguous. The entire A2 molecule is well resolved and fits the density well as evidenced by an average real space correlation coefficient of 83.8%. The positions of the termini of the GP2 peptide are also unambiguous and never altered through the course of refinement. However, unlike all reported pMHC structures, the center of the peptide never became clear in the density (Fig. 2). In addition, standard $2F_o - F_c$ maps, simulated annealing omit maps, unaveraged omit maps, and composite omit maps failed to show density for the center of the peptide. In particular, the orientation of residue 6 (Val) is completely uninterpretable, and the positions of residues 5 and 7 (Val and Gly, respectively) are not well defined.

## DISCUSSION

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

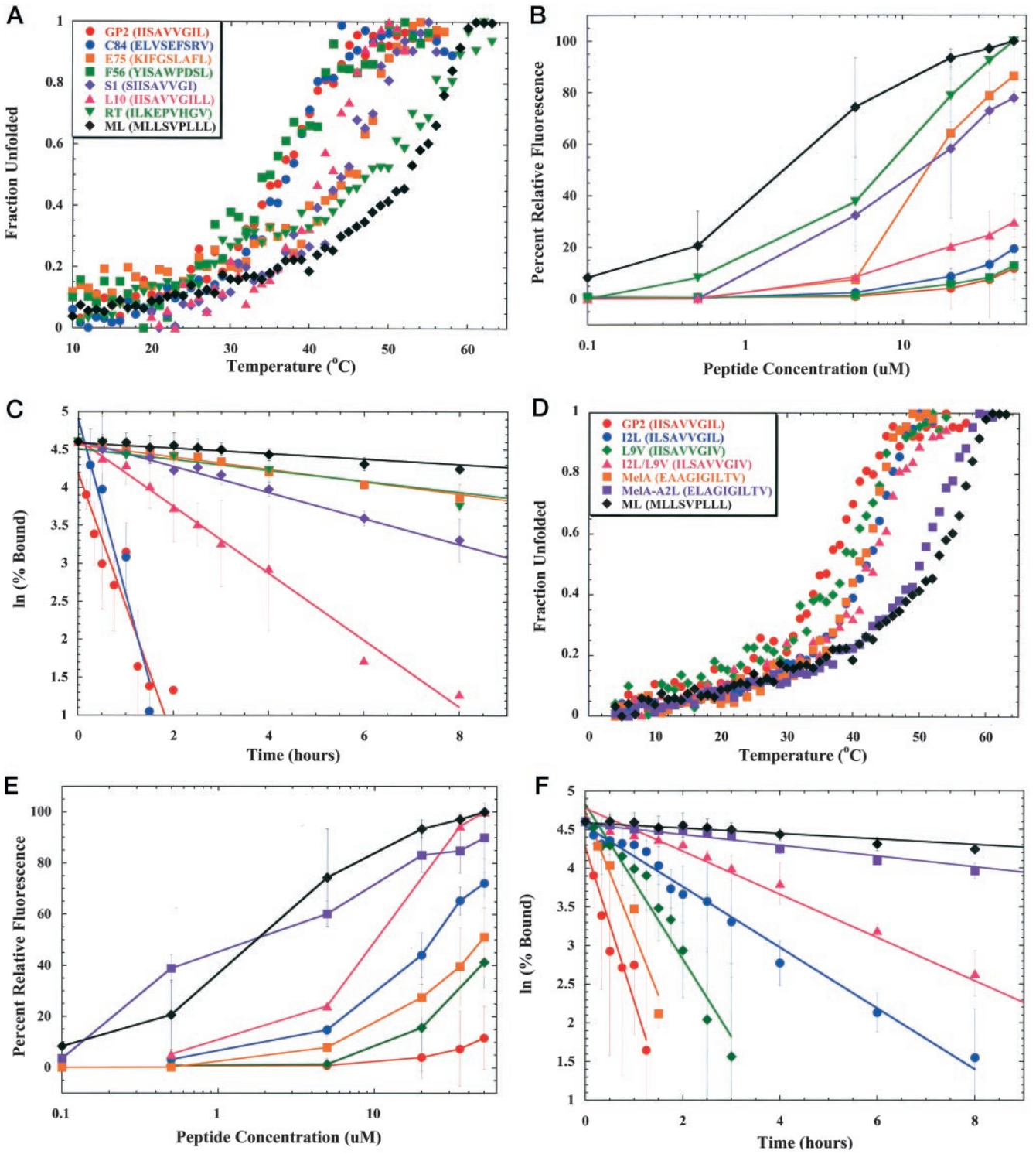


FIG. 1. **HER-2/neu-derived peptides bind poorly to A2, and anchor substitutions do not increase the stability of GP2-derived APL.** The symbols and colors shown in *A* are also those used in *B* and *C*. Likewise, the symbols and colors shown in *D* are also those used in *E* and *F*. *A*, thermal stability of A2-peptide complexes as measured by CD. 4–12 $\mu$M 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 $T_m$ 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.

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

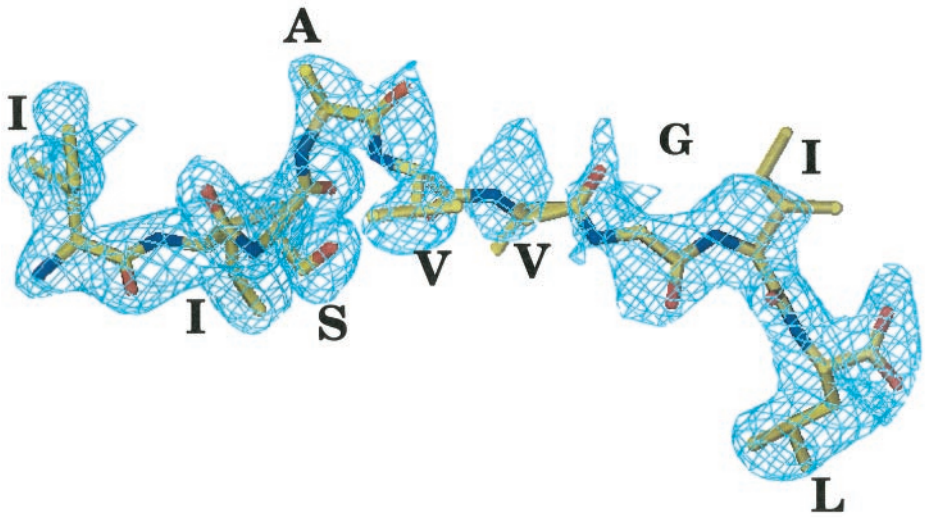


FIG. 2. **The center of the GP2 peptide is disordered.** The averaged omit electron density map of the GP2 peptide with a cover radius of 1.5 Å. The map was calculated using modified phases from DM.

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, C84, 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 crystal structures 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 would be able to recognize the complex well. Presumably, this set of T cells would be deleted from the T cell receptor repertoire, and they would not be in the periphery. If the self-peptide binds with poor affinity, the concentration of that peptide-MHC molecule in the thymus may be too low for recognition during the selection process. Therefore, there is a greater probability of finding these T cells in the periphery.

A complex of A2 with GP2 bound has poor thermal stability ($T_m$, 36.4 °C) and a very short cell surface half-life (~21 min). Many laboratories including ours have improved the binding affinity of peptides by changing the anchor residues to those most preferred by A2 (13, 36). As an example, we show that a small change in the anchor position of the melanoma peptide MelA results in a peptide with much greater binding affinity. However, trials with substitutions of GP2 at the anchor positions showed that the affinity was not significantly improved (Table I). Our goal is to be able to design APL for cancer immunotherapy. To be able to do this in a reasonable fashion, we needed to determine the crystallographic structure of A2-GP2 and determine why this peptide binds poorly.

The crystallographic structure shows uninterpretable electron density within the center of the peptide. Our interpretation of these data is that the peptide does not assume one unique conformation in the center as has been seen for all other single peptide-MHC structures to date (reviewed in Ref. 4). Interestingly, this is analogous to the situation found in the crystal structure of a class I MHC complex that contained a mixture of many different peptides (37). These data suggest that anchor substitutions do not significantly increase the affinity of GP2 because they do not address the fundamental problem that the peptide has in binding. The center does not make stabilizing contacts with the binding cleft of the class I MHC molecule.

This result begs another important immunological question. Does the flexibility in the center of the peptide increase or decrease immunogenicity? On the one hand, the flexibility decreases the already small concentration of a specific molecular surface that can interact with the T cell receptor on a circulating T cell. On the other hand, multiple peptide conformations generate more molecular surfaces that can be potentially recognized by circulating T cells. Perhaps in the context of a peptide that binds well, increased flexibility is more immunogenic, but in the context of a poor binding peptide increased flexibility does not increase immunogenicity because of the reduced concentration effect.

There is increasing interest in using peptides that bind to class I MHC for immunotherapy. As is the case of vaccination used to prevent viral infection, the potential therapeutic value is significant. As more antigens are discovered that are recognized by CTL and yet bind poorly to class I MHC molecules, the rules that predict binding affinity will be more critical. The phenomena observed here for GP2 certainly applies to other poor binding peptides whose binding affinity is not increased by altering the anchor residues. Increased affinity can be obtained for many of these peptides, but a full understanding of how peptides bind to class I MHC is still needed. By examining the binding of GP2 at the atomic level, we have made another step toward understanding peptide binding well enough to make predictions that will increase peptide affinity and minimize immunological consequences.

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REFERENCES

1. Lehner, P. J., and Trowsdale, J. (1998) *Curr. Biol.* **8,** R605—R608
2. Ploegh, H. L. (1998) *Science* **280,** 248–253

3. Peoples, G. E., Goedegebuure, P. S., Smith, R., Linehan, D. C., Yoshino, I., and Eberlein, T. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 432–436
4. Batalia, M. A., and Collins, E. J. (1997) *Biopoly* **43,** 281–302
5. Madden, D. R., Gorga, J. C., Strominger, J. L., and Wiley, D. C. (1992) *Cell* **70,** 1035–1048
6. Falk, K., Rotzchke, O., Stevanovic, S., Jung, G., and Rammensee, H.-G. (1991) *Nature* **351,** 290–296
7. Garrett, T. P. J., Saper, M. A., Bjorkman, P. J., Strominger, J. L., and Wiley, D. C. (1989) *Nature* **341,** 692–696
8. Rammensee, H. G., Friede, T., and Stevanovic, S. (1995) *Immunogenetics* **41,** 178–228
9. Colbert, R. A., Rowland-Jones, S. L., McMichael, A. J., and Frelinger, J. A. (1994) *Immunity* **1,** 121–130
10. Ruppert, J., Sidney, J., Celis, E., Kubo, R. T., Grey, H. M., and Sette, A. (1993) *Cell* **74,** 929–937
11. Pogue, R. R., Eron, J., Frelinger, J., and Matsui, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8166–8170
12. Bouvier, M., and Wiley, D. C. (1994) *Science* **265,** 398–402
13. Overwijk, W. W., Tsung, A., Irvine, K. R., Parkhurst, M. R., Goletz, T. J., Tsung, K., Carroll, M. W., Liu, C., Moss, B., Rosenberg, S. A., and Restifo, N. P. (1998) *J. Exp. Med.* **188,** 277–286
14. Ross, J. S., and Fletcher, J. A. (1998) *Stem Cells* **16,** 413–428
15. Fisk, B., Blevins, T. L., Wharton, J. T., and Ioannides, C. G. (1995) *J. Exp. Med.* **181,** 2109–2117
16. Yoshino, I., Goedegebuure, P. S., Peoples, G. E., Parikh, A. S., DiMaio, J. M., Lyerly, H. K., Gazdar, A. F., and Eberlein, T. J. (1994) *Cancer Res.* **54,** 3387–3390
17. Garboczi, D. N., Hung, D. T., and Wiley, D. C. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 3429–3433
18. Parham, P., and Brodsky, F. M. (1981) *Human Immunol.* **3,** 277–299
19. Zhao, R., Loftus, D., Appella, E., and Collins, E. J. (1999) *J. Exp. Med.* **189,** 359–370
20. Otwinowski, Z., and Minor, W. (1996) in *Methods Enzymol.* **276,** 307–326
21. Navaza, J., and Saludjian, P. (1998) *Methods Enzymol.* **276,** 581–594
22. Dodson, E. J., Winn, M., and Ralph, A. (1998) *Methods Enzymol.* **277,** 620–633
23. Madden, D. R., Garboczi, D. N., and Wiley, D. C. (1993) *Cell* **75,** 693–708
24. Brunger, A. T. (1992) *Nature* **355,** 472–474
25. Pannu, N. S., and Reed, R. J. (1996) *Acta Crystallogr. Sect. A* **52,** 659–668
26. Adams, P. D., Pannu, N. S., Read, R. J., and Brunger, A. T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5018–5023
27. Jones, T. A., Zou, J.-Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr. Sect. A* **47,** 110–119
28. Lamzin, V. S., and Wilson, K. S. (1998) *Methods Enzymol.* **277,** 269–305
29. Morgan, C. S., Holton, J. M., Olafson, B. D., Bjorkman, P. J., and Mayo, S. L. (1997) *Protein Sci.* **6,** 1771–1773
30. Chen, Y., Sidney, J., Southwood, S., Cox, A. L., Sakaguchi, K., Henderson, R. A., Appella, E., Hunt, D. F., Sette, A., and Engelhard, V. H. (1994) *J. Immunol.* **152,** 2874–2881
31. Tsomides, T. J., Walker, B. D., and Eisen, H. N. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 11276–11280
32. Lanzavecchia, A., Lezzi, G., and Viola, A. (1999) *Cell* **96,** 1–4
33. Brunger, A. (1992) *X-PLOR*, version 3.1, Yale University Press, New Haven, CT
34. Deleted in proof
35. Chan, S., Correia-Neves, M., Benoist, C., and Mathis, D. (1998) *Immunol. Rev.* **165,** 195–207
36. Romero, P., Gervois, N., Schneider, J., Escobar, P., Valmori, D., Pannetier, C., Steinle, A., Wolfel, T., Lienard, D., Brichard, V., van Pel, A., Jotereau, F., and Cerottini, J. C. (1997) *J. Immunol.* **159,** 2366–2374
37. Guo, H. C., Jardetzky, T. S., Garrett, T. P., Lane, W. S., Strominger, J. L., and Wiley, D. C. (1992) *Nature* **360,** 364–366
38. Kono, K., Rongcun, Y., Charo, J., Ichihara, F., Celis, E., Sette, A., Appella, E., Sekikawa, T., Matsumoto, Y., and Kiessling, R. (1998) *Int. J. Cancer* **78,** 202–208
39. Fisk, B., Savary, C., Hudson, J. M., O'Brian, C. A., Murray, J. L., Wharton, J. T., and Ioannides, C. G. (1995) *J. Immunother. Emphasis Tumor Immunol.* **18,** 197–209