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Modification of a Tumor-Derived Peptide at an HLA-A2 Anchor Residue Can Alter the Conformation of the MHC-Peptide Complex: Probing with TCR-Like Recombinant Antibodies

Galit Denkberg, Eynav Klechevsky, and Yoram Reiter

A common assumption about peptide binding to the class I MHC complex is that each residue in the peptide binds independently. Based on this assumption, modifications in class I MHC anchor positions were used to improve the binding properties of low-affinity peptides (termed altered peptide ligands), especially in the case when tumor-associated peptides are used for immunotherapy. Using a new molecular tool in the form of recombinant Abs endowed with Ag-specific MHC-restricted specificity of T cells, we show that changes in the identity of anchor residues may have significant effects, such as altering the conformation of the peptide-MHC complex, and as a consequence, may affect the TCR-contacting residues. We herein demonstrate that the binding of TCR-like recombinant Abs, specific for the melanoma differentiation Ag gp100 T cell epitope G9-209, is entirely dependent on the identity of a single peptide anchor residue at position 2. An example is shown in which TCR-like Abs can recognize the specific complex only when a modified peptide, G9-209-2 M, with improved affinity to HLA-A2 was used, but not with the unmodified natural peptide. Importantly, these results demonstrate, using a novel molecular tool, that modifications at anchor residues can dramatically influence the conformation of the MHC peptide groove and thus may have a profound effect on TCR interactions. Moreover, these results may have important implications in designing modifications in peptides for cancer immunotherapy, because most such peptides studied are of low affinity. *The Journal of Immunology, 2002, 169: 4399–4407.*

The TCR on lymphocytes having antitumor activity is able to recognize a unique structure on the tumor’s cell surface consisting of a peptide nestled in the groove of a surface MHC molecule (1–6). CD8+ T cells recognize peptides attached to class I molecules. Most class I-presented peptides are derived from the degradation of cytosolic proteins by a multienzyme complex in the cytoplasm called the proteosome. Cleaved peptides are transported into the lumen of the endoplasmic reticulum (ER) by a transporter associated with a processing molecule, possibly protect by chaperone heat-shock proteins from complete degradation before entering the ER. The trimmed peptides are bound to the groove of the assembled class I molecule in the ER, and the complex is transported to the cell surface (1–6).

A cascade of events can influence which peptides are found on the cell surface, including 1) the presence of endogenous and exogenous proteins; 2) the appropriate degradation of these proteins in intracellular compartments; 3) the ability of the degraded peptides to bind in the groove of the particular HLA molecules; and 4) the successful transport of these molecules to the cell surface. The absence or dysfunction of any of these processes can lead to defective peptide presentation on the tumor cell surface.

The interaction of the three-dimensional structure of the peptide-binding groove of the class I (and II) molecules with the conformation of the associated peptide determines which peptides can bind to particular HLA molecules as well as the affinity of this interaction. Specific sites in the groove of HLA molecules (called anchor sites) play a major role in determining the binding of the peptide to the MHC molecule. Other amino acid residues are more involved in interacting with the TCR, leading to recognition of this peptide and the MHC complex (1–6). The analysis of known peptides recognized by T cells as well as the analysis of sequences of pooled peptides eluted from HMC molecules have helped in characterizing peptides associated with particular MHC molecules (5, 6). The relatively uniform length of these peptides (e.g., 8–10 aa for most class I molecules and slightly longer peptides for class II molecules) has enabled defining of these allele-specific motifs for most of the common class I (and II) molecules (1–6). From these and other studies, it was determined that position 2 was an anchor residue favoring a leucine or methionine, and position 9 was a second anchor residue favoring a binding of a valine or leucine to the HLA-A*0201 molecule. Other residues with a lesser degree of preferred binding to HLA-A2 were also found. Examples of predominantly anchor-binding residues for other HLA alleles were also identified (3, 5). Knowledge of these allele-specific motifs has facilitated the identification of the immunogenic peptides of tumor Ags (7, 8). Most tumor Ags that have been characterized were derived from melanomas (9–11). These studies revealed that in contrast to most immunogenic viral peptides that have high binding affinity for their corresponding MHC molecules, most of the
immunogenic melanoma and melanocyte differentiation Ag peptides have a moderate or relatively low affinity for the HLA-A*0201 molecule (12–15). For example, of the four immunodominant HLA-A*0201-restricted peptides derived from the MART and gp100 Ags, three of the four peptides have a suboptimal amino acid residue at anchor position two and the other peptide has a suboptimal residue at anchor position nine (15). It is likely that T cell precursors capable of binding peptides with high affinity for HLA-A*0201 are deleted during lymphocyte maturation in the thymus and that only those T cells with relatively weak recognition of peptides survive negative selection (16, 17). Thus, although a correlation has been demonstrated between immunogenicity and peptide-binding affinity to class I MHC molecules for peptides derived from viral Ags, this appears not to be the case for the immunogenicity of peptides derived from nonmutated self determinants (12–15). The relatively low binding affinity of the immunodominant self peptides suggested that amino acid substitutions at anchor residue positions could increase the binding affinity without interfering with peptide recognition, and thus increase the immunogenicity of the peptide (18, 19, 20, 21). For example, Parkhurst et al. (15) studied numerous synthetic peptides with one or two amino acid substitutions of gp100-derived peptides designed to increase the binding affinity to the HLA-A*0201 molecule (15). In the gp100-209–217 peptide, the relative binding affinity of the leucine modification instead of threonine at the second position resulted in a peptide with a 52-fold higher binding affinity than the native peptide and yet was recognized as well as the native peptide by a specific tumor-infiltrating lymphocyte (TIL). CTLs induced by in vitro sensitization using the modified peptide exhibited superior recognition of the native peptide compared with CTLS raised with the native peptide. Similarly, a peptide with a modification of methionine at the second position was more effective than the native peptide in inducing CTLS reactive with the native peptide in vitro.

Based on these in vitro studies, a pilot clinical protocol was performed in which HLA-A*0201 patients with metastatic melanoma were immunized with the gp100-209–217 (G9-209 M)-modified peptide (M substituted for T at the second anchor position, aa 210) (22, 23). Using a stringent in vitro assay comparing pre- and postimmunization samples after a single in vitro exposure to peptide, only two of eight patients immunized with the native peptide were successfully immunized compared with 10 of 11 patients immunized with the modified peptide (23).

Interestingly, recent studies demonstrated changes in the fine specificity of gp100-derived 209-reactive T cells in some patients, following their vaccination with the 209 M-modified peptide (24). Moreover, it was shown that some PBMC-derived cloids isolated from these patients recognize the G9-209 M-modified peptide, but not the native one (24). These results suggested that a modification at an MHC anchor position may influence the overall conformation of the MHC-peptide complex groove and that T cells may sometimes recognize these differences.

Recently, we were able to isolate a new class of human recombinant Abs that recognize tumor-associated MHC-peptide complexes with the same specificity as the TCR. Interestingly, they recognized the MHC-peptide complex only when the specific peptide was present in the complex, yet unlike TCRs, they did so with an affinity in the nanomolar range (25, 26). In fact, we were able to isolate a surprisingly large panel of these human recombinant Fab Abs that exhibit a characteristic TCR-like binding specificity to each of three gp100-derived epitopes. We isolated these from a large nonimmune repertoire of phage Fab Abs selected on recombinant HLA-A2 complexed with three common antigenic T cell HLA-A2-restricted epitopes derived from the melanoma differentiation Ag gp100. These TCR-like Abs recognize the native MHC-peptide complex expressed on the surface of APCs. Moreover, they can detect the specific MHC-peptide complexes on the surface of melanoma tumor cells (26). The gp100-derived 209-modified epitope, 209 M, was one of the peptides used to select such Abs. In this study, we describe the binding characteristics of these TCR-like Abs and show that a TCR-like Ab selected toward the 209 M-modified peptide was able to bind only to HLA-A2 complexes displaying the modified, but not the native peptide. In this way, we demonstrate that these Abs can be used as an excellent molecular tool to study the structural and functional consequences of modifying MHC anchor residue positions. Importantly, we show in this study using these TCR-like Abs as conformation-sensitive sensors that a modification in anchor position can dramatically influence the conformation of the HLA-A2-peptide complex molecule.

Materials and Methods

Preparation of biotinylated single-chain (sc) β2 microglobulin (β2m)-HLA/A2 (scMHC)/peptide complexes

scMHC/peptide complexes were produced by in vitro refolding of inclusion bodies produced in Escherichia coli as described (27). Briefly, a scMHC construct, in which the β2m and HLA-A2 genes are connected to each other by a flexible peptide linker, was designed to contain the BiA recognition sequence for site-specific biotinylation at the C terminus (scMHC-BiA). This construct is expressed in E. coli, and upon induction with isoprropyl β-D-thiogalactoside, intracellular inclusion bodies that contain large amounts of the recombinant protein accumulate. Inclusion bodies are purified, reduced, and subsequently refolded in a redox-shuffling buffer system (0.1 M Tris, 0.5 M arginine, 0.09 mM oxidized glutathione, pH 8.0) in the presence of a 5–10 molar excess of the antigentic peptides. Correctly folded MHC/peptide complexes were isolated and purified by anion exchange Q-Sepharose chromatography (Amersham Pharmacia Biotech, Piscataway, NJ). Filtration using Centricron-30 units (Amicon, Bedford, MA) was used to exchange the elution buffer with Tris-HCl (10 mM, pH 8.0) and concentrate the scMHC-peptide complex to 1 mg/ml for specific biotinylation using the BirA enzyme (Avidity, Denver, CO) as previously described (28, 29). Excess biotin was removed from biotinylated complexes using a G-25 desalting column. The homogeneity and purity of the scMHC-peptide complexes was analyzed by various biochemical means including SDS-PAGE, size exclusion chromatography, and ELISA as described previously (30). The biological function of the scMHC-peptide complexes was determined by the ability of tetramers to stain CTL lines and clones in a peptide-specific manner. The generation of the scMHC-peptide tetramers and CTL staining procedures have been previously described in detail (28).

Isolation of TCR-like Abs

A large human Fab library containing 3.7 × 1010 different Fab clones was used for the selection (30). Phages (1013) were first preincubated for 1 h at room temperature (RT) in PBS containing 2% nonfat dry milk with streptavidin-coated paramagnetic beads (200 μl; Dynal Biotech, Oslo, Norway) to deplete streptavidin binders. Streptavidin-coated paramagnetic beads (200 μl; Dynal Biotech) were also incubated in PBS + 2% milk for 1 h at RT. The remaining phages were subsequently incubated for 1 h with decreasing amounts of biotinylated scMHC-peptide complexes (500 nM for the first round and 100 nM for the following rounds). Streptavidin magnetic beads were added, and the mixture was incubated for 15 min with continuous rotation. A magnetic force was applied to pull down phages bound to biotinylated complexes. After 10 washes of the streptavidin-bound complexes with PBS/0.1% Tween 20 and two washes with PBS, the bound phages were eluted by incubation for 5 min with 1 ml of triethylamine (100 mM). The elution mixture was neutralized by the addition of 100 μl of Tris-HCl (1 M, pH 7.4) and diluted to infect E. coli T7 phage cells (OD600 = 0.5) for 30 min at 37°C. Bacteria were grown overnight at 30°C on 2YT plates containing 100 μg/ml ampicillin (2YT/A/G) and 2% glucose.

Colonies were collected from the plates in 2YT/A/G and diluted 1/100 in 50 ml of medium. Cells were grown to OD660 = 0.5, and M13KO7 helper phage (5 × 107 CFU) was added to 5 ml of the culture. After incubation at 37°C for 30 min, the cells were centrifuged, resuspended in 25 ml of 2YT, ampicillin (100 μg/ml), kanamycin (50 μg/ml), and grown overnight at 30°C. Phages were collected from culture supernatants and purified for the next round of panning by polyethylene glycol precipitation.
The diversity of the selected Abs was determined by DNA fingerprinting. The Fab DNA of different clones was PCR amplified using the primers pUC-reverse (5'-ACGGGATACAATTACACAGG-3') and fd-tet-seq2 (5'-TTTCTGCTTCTTCCAGACGTAGT-3'). The resulting PCR fragments were digested with BstNI (NEB, Beverly, MA) (2 h, 37°C) and analyzed by agarose gel electrophoresis.

Expression and purification of soluble recombinant Fab Abs

Soluble Fabs were purified from the periplasmic fraction of BL21 cells using the hexahistidine tag fused to the CH1 domain of the Fabs. We produced and analyzed two to four Fab clones for each complex, which were selected according to their specificity pattern as assayed by ELISA with pure peptide. An overnight starter culture of Fab specific clones was grown at 30°C. Cells were diluted 1/100 into 500 ml of 2YT/A/G, grown to OD600 = 0.8–1.0, and induced to express the recombinant Fab Ab by the addition of 1 mM isopropyl-β-D-thiogalactoside for 4 h at 30°C. The cells were centrifuged and the pellet was resuspended in 5 ml of a B-PER solution (Pierce, Rockford, IL) to release periplasmic content. After 30 min of rotated incubation at RT, the solution was centrifuged (15,000 rpm, 15 min) and the supernatant was incubated with 0.5 ml of prewashed TALON beads suspension (Clontech Laboratories, Palo Alto, CA) for 45 min at RT. The solution was applied onto a Bio-Rad disposable column (Bio-Rad, Hercules, CA), and after sedimentation, the beads were washed three times with 10 ml of PBS, 0.1% Tween 20 (pH 8.0). The bound Fabs were eluted using 0.5 ml of 100 mM imidazole in PBS. The eluted Fabs were dialyzed twice against PBS (overnight, 4°C) to remove residual imidazole. The homogeneity and purity of the purified Fabs were determined by analysis on nonreduced and reduced SDS-PAGE.

ELISA with purified Fab Abs

The binding specificity of individual phage clones and soluble Fab was determined by ELISA using biotinylated scMHC-peptide complexes. ELISA plates (Falcon; BD Biosciences, Franklin Lakes, NJ) were coated overnight with BSA-biotin (1 µg/well). After having been washed, the plates were incubated (1 h, RT) with streptavidin (1 µg/well), washed extensively, and further incubated (1 h, RT) with 0.5 µg of MHC/peptide complexes. Plates were blocked for 30 min at RT with PBS/2% skim milk, and subsequently were incubated for 1 h at RT with phage clones (10⁶ phages/well) or various concentrations of soluble purified Fab, and after washing, with 1/1000 HRP-conjugated anti-myc Ab. Detection was performed using tetramethylbenzidine reagent (Sigma-Aldrich, St. Louis, MO). The HLA-A2-restricted peptides used for specificity studies of the Fab phage clones or purified Fab Abs are as follows: gp100 (154): KT-WGQYWQV; gp100 (209): IMDQVPFSV; gp100 (280): YLEPQVPVT; MUC1: LLLTVLTVL; HTLV-1 (TAX): LLFGYPVYV; MART1 (27): DFLLV; p53 (149): STPPPGTRV. The Fab DNA of different clones was PCR amplified using the hexahistidine tag fused to the CH1 domain of the Fabs. We produced and analyzed two to four Fab clones for each complex, which were selected according to their specificity pattern as assayed by ELISA with pure peptide. An overnight starter culture of Fab specific clones was grown at 30°C. Cells were diluted 1/100 into 500 ml of 2YT/A/G, grown to OD600 = 0.8–1.0, and induced to express the recombinant Fab Ab by the addition of 1 mM isopropyl-β-D-thiogalactoside for 4 h at 30°C. The cells were centrifuged and the pellet was resuspended in 5 ml of a B-PER solution (Pierce, Rockford, IL) to release periplasmic content. After 30 min of rotated incubation at RT, the solution was centrifuged (15,000 rpm, 15 min) and the supernatant was incubated with 0.5 ml of prewashed TALON beads suspension (Clontech Laboratories, Palo Alto, CA) for 45 min at RT. The solution was applied onto a Bio-Rad disposable column (Bio-Rad, Hercules, CA), and after sedimentation, the beads were washed three times with 10 ml of PBS, 0.1% Tween 20 (pH 8.0). The bound Fabs were eluted using 0.5 ml of 100 mM imidazole in PBS. The eluted Fabs were dialyzed twice against PBS (overnight, 4°C) to remove residual imidazole. The homogeneity and purity of the purified Fabs were determined by analysis on nonreduced and reduced SDS-PAGE.

Flow cytometry

The B cell line RMAS-HHD, which is transfected with a sc βM-HLA-A2 gene (31), EBV-transformed B-lymphoblast JY cells, or tumor cells as indicated were used to determine the reactivity of the recombinant Fab Abs with cell surface-expressed HLA-A2/peptide complexes. About 10⁶ RMAS-HHD cells were washed twice with serum-free RPMI and incubated overnight at 26°C in medium containing 100 µM concentration of the peptide. JY cells were loaded with peptide (100 µM) at 37°C. The APCs were subsequently incubated at 37°C for 2–3 h to stabilize cell surface expression of MHC-peptide complexes. The cells were incubated for 60–90 min at 4°C with recombinant Fab Abs (10–100 µg/ml) in 100 µl. After three washes, the cells were incubated with FITC-labeled anti-human Fab (The Jackson Laboratory, Bar Harbor, ME). After a final wash, the cells were resuspended in ice-cold PBS.

Adherent tumor cells were harvested by trypsinization and resuspended in cold RPMI. All subsequent washes and incubations were performed in ice-cold RPMI. The resulting PCR fragments were digested with BstNI (NEB, Beverly, MA) (2 h, 37°C) and analyzed by agarose gel electrophoresis.

Results

Isolation of gp100-specific TCR-like Abs

Recently, we isolated a large panel of human recombinant Fab Abs that exhibit a characteristic MHC-restricted, peptide-dependent, TCR-like binding specificity to each of three major gp100-derived T cell epitopes (9, 10). These Abs were isolated from a very large naive repertoire (3.7 × 10¹⁰ independent clones) of human Fab Abs displayed on phage, selected on scHLA-A2 complexes containing gp100 peptides (26, 27, 28, 30). gp100 is recognized by many HLA-A2-restricted melanoma-reactive TILs that have been isolated from melanoma patients (9, 10, 15). Five T cell epitopes have been identified in gp100; three of them are common immunogenic epitopes recognized by CTLs derived from different patients (9, 10): G9-154 (KTWGQYWQV), G9-209 (ITDQVPFSV), and G9-280 (YLEPQVPVT). It has been demonstrated previously that peptides G9-209 and G9-280 can be modified at the MHC anchor positions 2 (in G9-209M) and 9 (in G9-280V) to improve the binding affinity to HLA-A2 (15). When screening the large phage display library, we isolated Fabs 1A9 and 2F1, which recognize the HLA-A2/G9-209M and HLA-A2/G9-280V MHC-peptide complexes, respectively, in a TCR-like manner. Thus, as shown in Fig. 1, A and B, these recombinant Fab Abs bound to the
Differential binding of gp100-specific TCR-like Abs is dependent on anchor residue

The Fab Abs 1A9 and 2F1 were isolated by selection on recombinant HLA-A2 complexes displaying the modified peptides G9-209M and G9-280V, respectively. When we tested the binding of these Abs by ELISA to HLA-A2 displaying the modified or the wild-type peptide, we observed an interesting phenomena, namely that Fab 1A9 binds only to the HLA-A2 complex displaying the wild-type peptide, we observed an interesting phenomena, namely that Fab 1A9 binds only to the HLA-A2 complex displaying the wild-type peptide G9-209, but not the modified G9-209M, but not the wild-type peptide G9-209 (Fig. 2A). The binding specificity of 1A9 is maintained since it did not bind to the control gp100-derived G9-280 and its modified form G9-280V (Fig. 2A). However, Fab 2F1, selected on HLA-A2 complexes displaying the modified G9-280V peptide, binds the modified as well as the native peptide (Fig. 2B), but not to complexes displaying the G9-209 or G9-209M epitopes. The differential binding of Fab 1A9 is not due to differences in the stability of the HLA-A2/peptide complex during the assay, because as shown (Fig. 2, C and D), the conformation-specific mAb w6/32, which binds HLA molecules only when folded correctly and when it contains peptide, bound to all four complexes with a similar intensity (Fig. 2C). Titration binding of mAb w6/32 to HLA-A2/peptide complexes generated with wild-type 209 and modified 209-2M peptide did not reveal any differences in complex stability as well (Fig. 2D).

The ELISA was performed using biotinylated recombinant HLA-peptide complexes immobilized to BSA-biotin-streptavidin-coated immunoplates. The BSA-biotin-streptavidin spacer enables the correct presentation of the complexes, which can be distorted by direct binding to plastic. The correct folding of the bound complexes and their stability during the binding assays are thus confirmed by their ability to react with similar intensities with the conformation-specific W6/32 mAb.

To demonstrate that the differential recognition of modified vs wild-type peptide by 1A9 can be observed also at the level of the native complexes, as displayed on the surface of cells, we tested the binding of 1A9 and 2F1 to APCs that display the wild-type or the modified peptides. Briefly, the murine TAP2-deficient RMA-S cells transfected with the human HLA-A2 gene in an sc format (HLA-A2.1/Db-β2m sc) (RMA-S-HHD cells) were loaded with the appropriate peptides, and the ability of the selected Fab Abs to bind to peptide-loaded cells was monitored by FACS. First, we confirmed a similar peptide-induced MHC stabilization of the TAP2 mutant RMA-S-HHD cells by the modified and wild-type gp100-derived peptides. This was determined by analyzing the reactivity of the conformation-specific anti-HLA Ab w6/32 and the anti-HLA-A2 mAb BB7.2 on cells loaded with modified or wild-type peptides using FACS (Fig. 3).
As shown in Fig. 3, A and B, the extent that wild-type and modified HLA-A2-peptide complexes derived from gp100 epitopes G9-209 and G9-280, respectively, are displayed was identical when probing with the conformation-specific monoclonal w6/32. Similar results were observed when the display of the wild-type and modified complexes for the two gp100-derived epitopes was monitored with BB7.2 specific for HLA-A2 (Fig. 3, C and D). These results indicate that the extent of display, of modified and wild-type gp100 epitopes, on peptide-stabilized RMAS-HHD cells is similar. Similar results were observed when the modified and wild-type peptides were loaded on the TAP+ JY APCs (data not shown).

Next, we tested the binding of Fabs 1A9 and 2F1 to cells loaded with the modified and wild-type peptides. We used the TAP-mutant RMAS-HHD cells as described above and also JY APCs, which are EBV-transformed B lymphoblast cells that express HLA-A2 and are TAP+. Consequently, displaying the exogenous peptide on these JY cells is facilitated by peptide exchange. Using this strategy, we obtained a mixture of exogenously and endogenously derived peptides presented on HLA-A2 that are displayed on the cell surface. As shown in Fig. 4A, Fab 1A9 binds to RMAS-HHD cells loaded with the G9-209M-modified peptide, but not to cells loaded with the wild-type G9-209 peptide. Similar results were observed when testing the binding of 1A9 to JY cells loaded with modified or wild-type peptide; more specifically, 1A9 bound to cells loaded with the modified but not the wild-type peptide (Fig. 4C). As shown in Fig. 3, the differential binding of 1A9 was not due to differences in the display of peptides (modified vs wild-type) on the surface of the cells, because both were displayed to the same extent. However, Fab 2F1 did not exhibit any differential binding and was able to bind the wild-type as well as the modified peptide on both RMAS-HHD (Fig. 4C) and JY cells (Fig. 4D).

These results demonstrate that modification of an anchor residue in the G9-209 gp100-derived epitope can dramatically influence the conformation of the MHC groove recognized by the TCR-like Fab 1A9. We also tested the binding of 1A9 and 2F1 to tumor cells that are pulsed with the gp100-derived peptides. We used the HLA-A2+ melanoma cell line FM3D for pulsing with peptide and the HLA-A2-negative KB3-1 cell line as a control (Fig. 5). As shown in Fig. 6, 1A9 binds to FM3D cells only when pulsed with the modified G9-209M peptide, but not to cells pulsed with the native G9-209 peptide (Fig. 6A). Moreover, 1A9 did not bind to HLA-A2-negative KB3-1 cells pulsed with either peptide (Fig. 6B). In contrast to 1A9, Fab 2F1 binds to FM3D cells that are pulsed with both the modified and the wild-type G9-209 peptide (Fig. 6C), but not to peptide-pulsed KB3-1 cells (Fig. 6D). These results further corroborate the differential binding of Fab 1A9 to the modified but not to the wild-type G9-209 epitope. Combined, these results demonstrate that altering the anchor position 2 of G9-209 can dramatically influence the binding of TCR-like Fabs as described in this study, which can be regarded as sensors to the conformation of the Ag peptide in the context of the MHC peptide-binding groove.

**Binding of gp100-specific tetramers to CTLs**

To demonstrate that the TCR-like Fab Abs can be used as sensors for monitoring conformational changes that cannot be detected by the native TCR due to differences in the biophysical nature of these two molecular entities, we tested the recognition of a CTL clone by...
scMHC tetramers generated with wild-type and modified peptides. We have shown previously that our scMHC tetramers, in which the human β2m gene is connected covalently to the three extracellular domains of HLA-A2 using a 15-aa-long flexible linker, are functional (27, 28). To determine whether the staining of CTL clones can be influenced by the peptide present in the HLA tetramers, we analyzed the staining pattern of two CTL clones derived from patients who were vaccinated with the gp100-derived modified epitopes G9-209M and G9-280V (32). As shown in Fig. 7, A–D, the CD8+ CTL clone R6C12 (Fig. 7A), specific for the G9-209M peptide, was stained intensively (80–90%) with scHLA-A2 tetramers containing the wild-type as well as the modified peptide (Fig. 7, B and C), but not with tetramers containing the G9-280 peptide (Fig. 7D). Similarly, the CD8+ CTL clone R1E2 (Fig. 7E) was stained intensively with scHLA-A2 tetramers containing the wild-type or modified G9-280 epitope, but not the G9-209 epitope.

The results indicate that these particular CD8+ CTL clones cannot differentiate between tetramers displaying the modified or the wild-type gp100-derived peptides.

Functional properties of anchor-specific TCR-like Abs

To further demonstrate the unique fine specificity of the anchor-specific TCR-like Fab Ab 1A9, we tested its ability to interfere with peptide-induced T cell activation of a gp100-specific T cell clone. The TAP mutant human T2 cells, which express HLA-A2, were reacted with scHLA-A2 tetramers generated with wild-type (B) as well as anchor-modified (C) G9-209 epitope, but not with tetramers containing the G9-280 L peptide. The CD8+ CTL clone R1/E2 (E), specific for the G9-280 L peptide, was reacted with wild-type (F) as well as anchor-modified G9-280 (G), but not with tetramers containing the peptide G9-209 (H).

FIGURE 7. Binding of gp100-derived peptide-HLA-A2 tetramers to gp100-specific CTL clones. Functional analysis of the ability of two CTL clones to bind scHLA-A2/gp100-derived tetramers. CD8+ CTL clone, R6C12 (A), specific for the gp100-derived peptide G9-209 in complex with HLA-A2, was reacted with scHLA-A2 tetramers generated with wild-type (B) as well as anchor-modified (C) G9-209 epitope, but not with tetramers containing the G9-280 L peptide. The CD8+ CTL clone R1/E2 (E), specific for the G9-280 L peptide, was reacted with wild-type (F) as well as anchor-modified G9-280 (G), but not with tetramers containing the peptide G9-209 (H).

FIGURE 8. Inhibition of T cell activation by TCR-like Fab 1A9. A, Peptide-specific T cell activation of G9-209-specific R6C12 CTL. T2 cells were pulsed with wild-type and anchor-modified G9-209 or control telomerase-derived T865 epitope. Peptide-specific CTL activation with G9-209 but not T865 is shown. Inhibition of T cell activation in the presence of Fab 1A9 (100 nM) was observed when T2 cells were pulsed with G9-209M-modified peptide, but not when pulsed with wild-type peptide. B, T2 cells were pulsed with wild-type (G9-209) or anchor-modified (G9-209M) peptide and incubated with the G9-209-specific HLA-A-restricted CTL clone R6C12 in the presence of various concentrations of Fab 1A9 as indicated. T cell stimulation was measured by the release of IFN-γ to the culture medium. IFN-γ was determined by a double sandwich ELISA. Fab 1A9 inhibited T cell activation in T2 cells pulsed with the anchor-modified G9-209M peptide, but not in cells pulsed with wild-type peptide.

Peptides bind to class I MHC primarily through the invariant peptideic termini (1–6, 33, 34). In addition, the polymorphic residues within the peptide-binding groove create specificity pockets that select specific amino acids in the peptide (35, 36). The specificity pockets for HLA-A2.1 (A2) are found close to the peptideic termini and are complementary in shape and charge to residues 2 (P2) and the last residue of the peptide (P9). The specificity pockets play a large role in binding affinity to HLA-A2 but not to all class I MHCs (37–39). The result of this set of interactions in HLA-A2 is the binding of the ends of the peptide, leaving the center relatively free of interactions. The center of the peptide bulges out of the peptide-binding cleft, and the main chain rarely traverses the same.
path in two different peptides. The cocystal structures of class I MHC and TCR show that the means of engagement between peptide-MHC and TCR are conserved (40-44). The TCR binds in a diagonal manner with the TCR α-chain, interacting with the carboxyl end of the MHC α2 helix, and the TCR β-chain interacting with the carboxyl end of the MHC α1 helix. The CDR3 regions of the TCR α and β-chains interact with the center of the peptide (P5-P7 depending on the peptide) (45, 46).

Two characteristics about peptide binding to class I MHC are revealed by these structural informations. The first is that we have assumed that each amino acid in the peptide binds independently of one another to enhance or detract from the overall binding affinity. Considering this assumption, a popular algorithm was designed to predict peptide epitopes that bind well. This algorithm is based on the assumption that each residue binds independently (47). Although this algorithm predicts many good binding peptides from proteins of interest (48-51), for unknown reasons it fails to predict accurately the results of single amino acid substitutions.

The second characteristic of peptide binding explored in this study is that residues at the anchor positions do not affect the conformation of residues elsewhere in the peptide. If one assumes that each residue binds independently, homologous substitutions would be the best choice to amplify and activate T cells specific for the parental Ag (20, 21). The clear choice for modification is the anchor residues, because they point into the binding cleft and are restricted in space by the specificity pockets. Thus, the conformation flexibility of the peptide backbone should be limited, and any alterations in the structure caused by the anchor substitution would be expected to be local and small. In light of this consideration, peptides (termed altered peptide ligands) have been designed with increased affinity for class I MHC to enhance CTL stimulation (15, 20, 21, 23, 52, 53).

In this study, we have demonstrated that substitution in the anchor residues can dramatically alter the overall conformation of the peptide-MHC complex. Using a new molecular tool in the form of a recombinant Ab endowed with the Ag-specific MHC-restricted specificity of a TCR (TCR-like Ab), we have shown that the Ab can distinguish between peptide-MHC complexes generated with a modified anchor residue. TCR-like Abs are excellent molecules for use as probes for MHC-peptide groove conformation. Peptides bound to MHC are deeply buried in the MHC and are presented as peptide contact residues. Strikingly, no >100-300 Å² of MHC class I-bound peptide faces outwards, and thus is available for direct recognition. The MHC is thought to account for most of the peptide-MHC complex surface presented to T cells. Abs recognizing protein molecules engage ~800 Å² of their ligand (54) so that Ab recognition of a peptide-MHC complex (even if peptide-specific) would also have to be dominated by the overall MHC conformation. Therefore, the surface area recognition of a TCR-like Ab must encompass both the peptide and MHC. Indeed, as shown in this study, such an Ab can be used to detect conformational changes caused by modification of the peptide anchor position.

Further evidence supporting this came recently from a structural study demonstrating that class I MHC anchor substitution alters the conformation of TCR contacts (55). In this study, the binding to the class I MHC molecule HLA-A2 of a selection of known immunologically recognized peptide ligands from the tyrosine kinase family member HER-2/neu was examined.

Crystallographic analysis of these MHC complexes generated with modified peptides revealed that the TCR contact residues alter their positions depending on the identity of the anchor residue (55). The data presented show that substitutions in the center of a peptide bound to class I MHC may affect the positions of all of the residues within the peptide. In addition, small homologous substitutions in the anchor residues can dramatically alter the TCR-contacting residues. Clearly, the presence of substituted residues may alter the positions of nearly all of the other residues even when the substitution is a minor homologous substitution. The structural data in this study on the effect of modifying anchor positions is markedly enhanced by the data we have presented in this study demonstrating that the binding of a conformation-specific Ab with TCR-like properties is entirely dependent on the identity of a single anchor position.

An interesting question is how this can influence reactivity with T cells. T cells stimulated using altered peptide ligands are not necessarily the same population of T cells. Our study provides an explanation for the instances in which alteration of anchor position reduces or eliminates reactivity with T cells. The data presented by Sharma et al. (55) show that substitutions at the anchor positions can directly alter the conformation of the residues at the center of the peptide and conversely, that substitutions in the center can cause significant changes at the termini.

In the HLA-A2/G9-209 complex, position 2 is an HLA-A*0201 anchor residue that is not considered a TCR contact residue. However, the data presented in this study suggest that an HLA-A2 molecule containing the G9-209-2M peptide has a different conformation than an HLA-A2 molecule containing the wild-type parent G9-209 peptide. These conformational differences could be detected by the TCR-like G9-209-2M-specific Fab Ab 1A9. Importantly, these results indicate that modification of MHC binding anchor residues that do not have contact with the TCR can influence the overall conformation of the MHC peptide groove. The CTL clone chosen to test this in our study was not able to differentiate between the modified and the wild-type G9-209 peptide. However, in a recent study that analyzed the reactivity of TILs and T cell clones derived from PBMC of patients vaccinated with G9-209-2M, changes in the fine specificity of the gp100 G9-209-reactive T cells were observed (24). It was found that immunization with the modified G9-209-2M peptide may have skewed the anti-G9-209 immune response in the vaccinated patients towards T cells with a reduced capacity to recognize tumor cells. In fact, the majority of peptide-reactive clones did not recognize tumors. An interesting observation with respect to TCR recognition and differentiation between the wild-type and modified peptide was that several of the PBMC clones were G9-209-2M-specific, and thus were not expected to be tumor-reactive because they do not recognize the parental G9-209 peptide. Other modifications in position 2 were also not recognized by these clones.

This observation further strengthens our observation that modification of the anchor position 2 in G9-209, which is not considered a TCR contact residue, can influence the conformation of the MHC-peptide complex. The interesting findings by Clay et al. (24) suggest that T cells are capable of detecting these differences and that conformational changes induced by MHC binding anchor residues that do not contact the TCR could influence T cell reactivity. Further support for these observations came from studies using T cell clones that are specific for the same parental peptide in which altered peptide ligands resulted in differences in the T cell activation state among the different T cell clones.

In summary, the data presented in this study show that substitutions in anchor residues can dramatically alter the conformation of the peptide-MHC complex, and thus may have an effect on TCR-contacting residues. This may have important implications in cancer immunotherapy because most peptides studied are of low affinity; and therefore, modifications of anchor position are used to design peptides with increased affinity for class I MHC to enhance CTL stimulation.
References


