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Anti-MUC1 Antibodies React Directly with MUC1 Peptides Presented by Class I H2 and HLA Molecules

Vasso Apostolopoulos,* Gareth Chelvanayagam,[†] Pei-Xiang Xing,* and Ian F. C. McKenzie[1]*

Peptides bound in the groove of MHC class I molecules and detected by CTLs are not normally accessible to Ab. We now report that MUC1 peptides that are bound within the groove of MHC class I molecules (H2 and HLA) and that can be detected by CTLs can also be detected by anti-MUC1 Abs. mAbs to the middle and C-terminal regions of the class I-associated peptides but not to the N terminus were able to react with MUC1 peptides bound to H2K[b] and HLA-A*0201, and only to the mid-region for H2D[b], by flow cytometry and also to block CTL activity. Molecular modeling showed that the N terminus is buried (and not accessible), whereas the midpeptide residues form a loop and the C terminus is free, making these two regions accessible to Ab. The findings demonstrate for the first time that peptides associated with class I molecules can be detected by anti-peptide Abs. *The Journal of Immunology,* **1998, 161: 767–775.**

C D8[+] CTLs recognize short peptides associated with MHC class I molecules, and while it has been difficult to induce CTLs to exogenous peptides by immunization, they have been described after viral infections such as influenza and EBV (1–4); in human melanoma, the activity of CTLs is restricted to HLA-A1 and HLA-A*0201 alleles (5). In breast cancer, it is possible that mucins (MUC1) could be a suitable target for immunotherapy (6), and there is now a major focus on the induction of effective CTLs in cancer for therapeutic purposes.

Recent structural studies, including crystallization of class I molecules of H2 and HLA molecules with bound peptides, have delineated how peptides bind within the groove of class I molecules. Peptides of 8 to 10 amino acids, usually with defined anchor motifs, bind with high affinity (7); however, peptides lacking such motifs (8–10), including MUC1, can also bind to class I molecules, albeit with low affinity, and can be detected by high avidity anti-MUC1 CTLs (11). The structural studies performed thus far indicate that the class I-presented peptides are buried within the groove of class I, leaving little of the peptide exposed for the direct interaction of class I and peptide with the Ig-like T cell $\alpha\beta$ receptor. Peptides presented by mouse H2 (12, 13) or human HLA (14–18) molecules have revealed a number of striking similarities in that: 1) the peptides are anchored in the cleft by amino acids binding in specific pockets; 2) the mode of peptide binding appears to be canonical, irrespective of whether the peptide is 8, 9, or 10 amino acids in length, and the main chain conformation at the ends of the peptides is essentially the same; and 3) the central portion of the peptide bulges out of the cleft (12, 14–18) and can be detected by CTLs. The binding sites on the TCR are imposed by the shape of the complementarity-determining regions (CDRs) that recognize the homologous site of the peptides and the edges of the class I groove. Given the structural similarity of TCR and Ig complementarity-determing regions, it is not surprising that Abs have been described that bind with contiguous amino acids in class I and bound peptide (i.e., MHC-peptide specific (19)) or with the unique configuration of the peptide imposed by the class I groove (20). The lack of Ab reactivity with peptides in the groove using Abs that react with the linear structure of "free" (non-class I-bound) peptides has been explained by the suggestion that the sequences of the peptides are hidden within the class I groove. To date, no study has matched Ab-binding sites in a free peptide with the same sites in the peptide bound to class I molecules.

We now describe MUC1 Abs, which react with linear peptides (in the absence of class I molecules) and which also detect MUC1 peptides while bound in the groove of class I molecules. These findings suggest that peptides that "loop" out of the groove, or in which the C-terminal end is free (i.e., not anchored), can be accessible to such Abs. Abs targeted to epitope residues that are anchored and therefore buried (e.g., N-terminal) do not react with the peptide in the groove. Molecular modeling, used to provide a rational explanation for the observations, shows that peptides bind in a nonconventional manner, particularly at the C-terminal end, leading to their exposure and availability for Ab binding.

Materials and Methods

Target cells and peptide loading

MHC class I molecules in the murine cell line RMA-S (C57BL/6 TAP-deficient cells) are unable to engage endogenous peptides. Empty class I molecules formed in this cell line are unstable at physiologic conditions (37°C) and are not expressed in sufficient amounts to be detected by anti-class I Abs, but are expressed in considerable quantities at the surface at reduced temperatures (26°C) or if cultured with a "presentable" peptide. This distinction can be used to measure the direct binding of peptides to class I molecules. RMA-S cells were incubated overnight with 20-μM 9-mer peptides at 26°C for 3 h and were used either in flow cytometry (see below) or as targets in a CTL assay. For competitive binding of MUC1 peptides, RMA-S cells were pulsed with 20 μM K[b] (SAPDTRPAP) peptide and increasing amounts SIINFEKL (0–20 μM) for 3 h and then stained by anti-MUC1 peptide Abs. Percentage of binding of Abs to cells was calculated as the percentage of Ab binding to cells pulsed with peptide − percentage of Ab binding negative control. In addition, HLA-A*0201 EBV-immortalized B cells pulsed with 20 μM MUC1 peptide were used as targets or for direct binding of anti-MUC1 mAbs by flow cytometry.

To prepare PHA (PHA-L; leucoagglutinin, Sigma, St. Louis, MO) blast cells, 2×10^6 C57BL/6, or class I-deficient β_2-microglobulin mouse spleen cells were placed into wells of a 24-well plate with 1 μg/ml PHA and incubated for 2 days at 37°C in 10% CO_2 to form blast cells. PHA blast

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Table I. *Anti-MUC1 reactions with MUC1 peptides presented by H2K[b], H2D[b], or HLA-A*0201[a]*

Ab	Mucin 1 Peptide	Reaction with Peptide		CTL Blocked	Predicted Binding (model)
		Bound in groove	On pins		
K[b] peptide	**S A P D T R P A P** G S T A P P A				
BC2	A P D T R	−	+	−	−
VA1	A P G	−	−	−	−
VA2	D T R P A	+	+	+	+(?R)
BCP8	D T R	+	+	+	+(?R)
BCP9	G S T A P	−	−	−	−
BCP10	R P A P	+	+	+	+
D[b] peptide	S A P D T R P **A P G S T A P P A**				
BC2	A P D T R	−	−	−	−
VA1	A P G	−	+	NT	−
VA2	D T R P A	−	−	NT	−
BCP8	D T R	−	−	−	−
BCP9	G S T A P	+	+	+	+(?T)
BCP10	R P A P	−	−	NT	−
HLA-A*0201 peptides	**S A P D T R P A P** G S T A P P A				
BC2	A P D T R	−	+	−	−
VA1	A P G	−	−	NT	−
VA2	D T R P A	+	+	+	+
BCP8	D T R	+	+	+	+
BCP9	G S T A P	−	−	−	−
BCP10	R P A P	+	+	+	+

[a] − Indicates negative; +, positive; NT, not tested; peptides (class I binding peptide or on pins) are in bold; ?R and ?T refer to the uncertain role of these amino acids in the epitope.

cells were then incubated overnight with 20 μM MUC1 peptides and tested for the ability of anti-MUC1 Abs to bind by flow cytometry. We optimized the culture conditions that generated low spontaneous release of ^{51}Cr and high levels of expression of MHC class I molecules from PHA blast cells.

CTL assay

Spleen cells from C57BL/6 and HLA-A*0201 transgenic mice, immunized i.p. with M-FP (5 μg of the 39-kDa MUC1 fusion protein coupled to oxidized mannan), were obtained 7 days after the third immunization, washed in PBS containing 2% FCS, and used directly in a ^{51}Cr release assay (6, 21, 22). Cells were radiolabeled with 100 μCi of Na$_2$^{51}CrO$_4$ (^{51}Cr) (Amersham, Arlington Heights, IL) for 60 min at 37°C. For inhibition assays, target cells were preincubated with various dilutions of Abs for ~15 min at room temperature before being added to effector cells. A constant E:T ratio was used at 100:1.

Abs and flow cytometry

The anti-MUC1 Abs used, which detect discrete MUC1 epitopes, were: VA1 (amino acids: APG), VA2 (amino acids: DTRPA), BC2 (amino acids: APDTR), BCP8 (amino acids: DTR), BCP9 (amino acids: GSTAP), BCP10 (amino acids: RPAP), and STAP31 (amino acids: AH) (Table I) (23–25). One hundred microliters of various dilutions of ascites (neat = ~1 mg/ml) was added to a pellet of 2×10^5 cells (RMA-S peptide-loaded cells, HLA-A*0201 EBV-immortalized B-cells, or splenocyte PHA blasts) and incubated for 1 h at 4°C. After washing with 0.5 ml phosphate buffer, 100 μl 1:50 dilution of FITC-conjugated sheep (Fab′)$_2$ anti-mouse Ig (Silenus, Melbourne, Australia) was added and incubated for 45 min at 4°C; after further washing, the cells were analyzed by flow cytometry using a FACScan flow cytometer.

Computer modeling

To investigate how the peptides SAPDTRPAP (H2K[b]), APGSTAPPA (H2D[b]), SAPDTRPAP, and STAPPAHGV (HLA-A*0201) might bind to class I molecules, these peptides were modeled into the Ag-binding clefts of H2K[b], H2D[b], and HLA-A*0201. modeling was performed with the HOMOLOGY, BUILDER, and DISCOVER programs from the Insight II package (Biosym, San Diego, CA) and made use of the crystal structures of H2K[b] complexed with the SEV peptide (FAPGNYPAL) (13), H2D[b] complexed with the influenza virus nucleoprotein (NP)[2] peptide NP$_{366-374}$ (ASNENMDAM) (26) and HLA-A*0201 complexed with the LLF-

GYPVYV (HTLV-1 tax$_{11-19}$) and ILKEPVHGV (HIV-1 RT$_{276-484}$) peptides (17) as templates. The STAPPAHGV peptide was modeled using the ILKEPVHGV template, while the SAPDTRPAP peptide was modeled using both templates, since this allowed an initial conformation of R (P6) to point either into or out of the cleft). Initially, each crystal structure was truncated at residue 185 with the C-terminal residue protected with an *N*-methyl group; hydrogen atoms were then introduced with the BUILDER program. Energy minimization and dynamics were then performed as previously described (18).

Results

We had previously shown that immunization of mice with either MUC1$^+$ tumors or MUC1 fusion protein conjugated to oxidized mannan (M-FP) generated CD8$^+$ CTLs that recognize both MUC1 transfected and peptide-pulsed target cells, in a MHC class I (H2/HLA)-restricted manner (21, 22, 27, 28), the antigenic epitopes being presented by H2 D, L, or K class I molecules of the *b*, *d*, *k*, *s*, and *z* haplotypes or by HLA-A*0201 in transgenic mice and by human HLA-A*0201, MUC1$^+$ tumor cells. In addition, the epitopes presented by six different class I alleles (H2K[b], D[b], D[d], L[d], K[k], and HLA-A*0201), which do not contain the known anchor motifs, were identified (11, 29): the peptide SAPDTRPAP was presented by H2K[b]; APGSTAPPA was presented by H2D[b]; and SAPDTRPAP and STAPPAHGV were both presented by HLA-A*0201. Here, we describe a novel feature of the association of MUC1 peptides with H2 and HLA molecules in that the class I-associated MUC1 peptides can be recognized by MUC1 antipeptide Abs, which also react with the free peptides. The panel of anti-MUC1 peptide Abs used in this study has been described previously (23–25); these are listed in Table I.

Anti-MUC1 peptide Abs block class I-restricted CTL activity

The peptide SAPDTRPAP is presented by H2K[b] molecules and, using Abs that identify different sites on this peptide (BC2, VA2, BCP8, and BCP10) (Table I), it was possible to block CTL lysis of MUC1$^+$ target cells by some of the Abs (VA2, BCP8, and BCP10) (Fig. 1*A*). There was no blocking by BC2 (to the N-terminal APDTR), however, Abs to the middle part of the peptide (DTR or

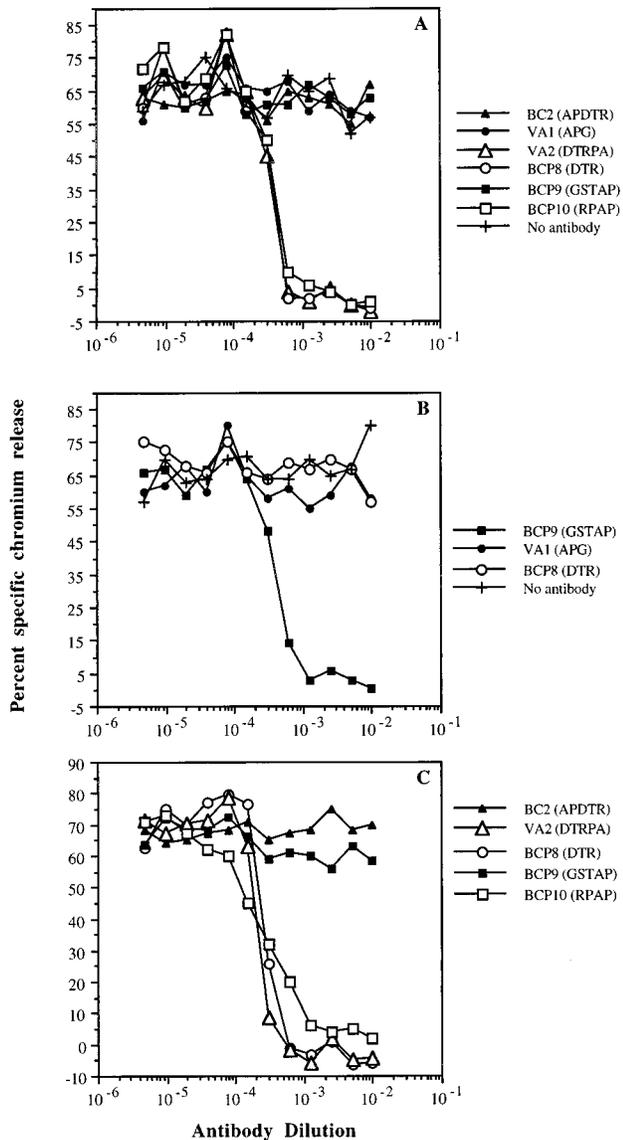[2] Abbreviations used in this paper: NP, nucleoprotein; HA, hemagglutinin.

FIGURE 1. Inhibition of CTL lysis by anti-MUC1 Abs. Before the addition of the C57BL/6 effector cells, ^{51}Cr RMA-S cells pulsed with the K^b-binding peptide SAPDTRPAP (A) or the D^b-binding peptide APG-STAPPA (B) were incubated with anti-MUC1 mAbs at dilutions of 1/100 to 1/32,000. C, Effector spleen cells from transgenic HLA-A*0201 mice were incubated with ^{51}Cr HLA-A*0201-EBV-B cells, pulsed with the SAPDTRPAP peptide, and preincubated with anti-MUC1 mAbs. A constant E:T ratio of 100:1 was used. Ordinate, percentage of specific chromium release; abscissa, Ab dilution.

FIGURE 2. Flow cytometric analysis of RMA-S cells pulsed with A, K^b (SAPDTRPAP)- and B, D^b (APGSTAPPA)-binding peptides before incubation with anti-MUC1 peptide Abs. Ordinate, cells per channel; abscissa, fluorescence intensity.

MUC1 peptides binding to HLA-A*0201 molecules were also accessible, because some anti-MUC1 Abs blocked CTL lysis of HLA-A*0201 cells (Fig. 1C). Four potentially relevant Abs were available for the examination of how epitopes in the bound peptide were accessible: BC2 (APDTR, N-terminal peptide); BCP8 (DTR, midpeptide); VA2 (DTRPA) and BCP10 (RPAP, C-terminal peptides); and BCP9 (GSTAP, negative Ab). Target cells (HLA-A*0201-EBV-B cells) were pulsed with SAPDTRPAP, separately incubated with the five Abs, and mixed with effector cells. It was found that Abs BCP8, BCP10, and VA2 inhibited CTL lysis to a similar degree (Fig. 1C, Table I), demonstrating the binding of the Ab to an accessible epitope, whereas BC2 and the nonreactive BCP9 had no effect. It appeared that the Abs were reacting directly with the peptides bound in the groove of the class I molecules, and subsequent studies were aimed at proving this hypothesis.

Ab detection of MUC1 peptides presented by H2K^b and H2D^b class I molecules

RMA-S cells were loaded with peptide, washed, incubated with anti-MUC1 Abs and analyzed by flow cytometry. When cells exposed to the K^b peptide (SAPDTRPAP) were tested using Abs that react with different parts of the 9 mer, the BC2 (APDTR), VA1 (APG), and BCP9 (GSTAP) Abs were nonreactive, but the Abs VA2 (DTRPA), BCP8 (DTR), and BCP10 (RPAP) bound to RMA-S cells (Fig. 2A; Table I). Thus, the amino acid residues at the N terminus (SAPDTR . . .) appeared to be inaccessible (no reaction with Ab BC2), whereas middle (. . . DTR . . .) and C terminal (. . . RPAP) amino acids could bind Abs. The BCP9 Ab to the nonbound GSTAP peptide did not react, providing a suitable specificity control.

DTRPA residues detected by BCP8 and VA2 Abs, respectively) could block CTL lysis, as could the BCP10 Ab to the C-terminal RPAP. There was no blocking by the anti-GSTAP Ab (BCP9) or anti-APG (VA1) Abs, which are not part of the class I-bound epitope and serve as controls (Fig. 1A). Thus, Abs to the mid- and C-terminal, but not the N-terminal, parts of the SAPDTRPAP peptide could block CTLs.

For the MUC1 peptide APGSTAPPA presented by H2D^b molecules, only one Ab (BCP9), which reacted to the central GSTAP region of the bound peptide, was able to block CTL lysis of MUC1$^+$ target cells (Fig. 1B). The Ab VA1 to the N terminus (APG) did not inhibit, nor did the isotype control Ab BCP8 (Fig. 1B, Table I).
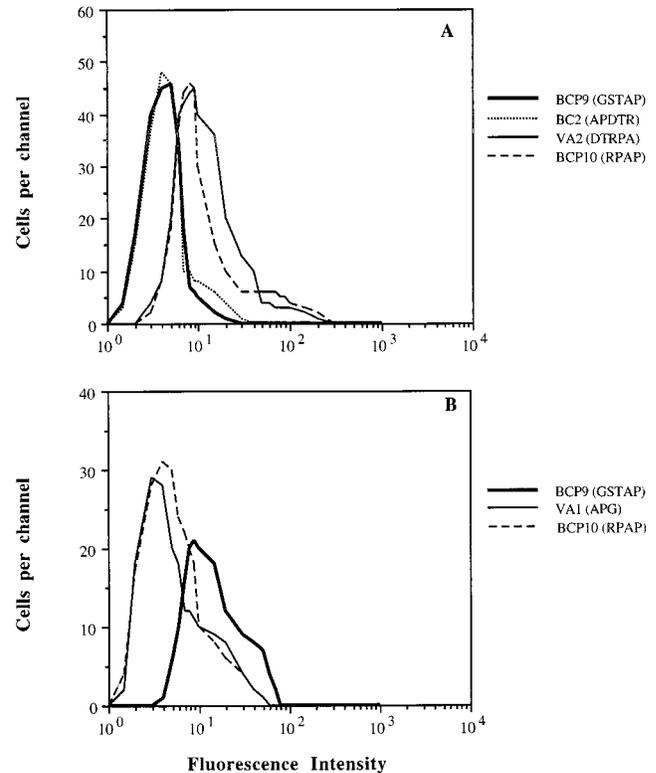
Similar results were obtained with a D^b peptide when RMA-S cells were loaded with the D^b MUC1 9 mer (APGSTAPPA). The Ab BCP9 (GSTAP) was reactive, whereas VA1 (APG), VA2 (DTRPA), BC2 (APDTR), BCP8 (DTR), and BCP10 (RPAP) Abs were not (Fig. 2B, Table I). Of the Abs used, only VA1 and BCP10 were potentially reactive, epitopes for other anti-VNTR Abs not being represented (22, 24). The finding that the VA1 Ab reacts with the N-terminal amino acids APG in the free state of the D^b peptide (APGSTAPPA) but do not stain peptide pulsed cells suggests that these amino acids are hidden in the structure of the peptide/MHC complex. By contrast, BCP9 (GSTAP) reacts with amino acids in the center of both the free peptide and the class I-bound peptide. This suggests that these sequences are available for recognition by the Ab, possibly by the looping of the peptide out of the class I groove, and modeling shows that this is likely to be the case (see below). It should be noted that none of the Abs bound to RMA-S cells pulsed with the MUC1 peptide APPAH GVTS, because it is not presented by class I molecules (not shown).

Similar results were found with C57BL/6 PHA blast splenocytes pulsed with peptide SAPDTRPAP (for K^b) or APGSTAPPA (for D^b), in that specific binding of anti-MUC1 Abs occurred, whereas when using peptide-pulsed inbred C57BL/6 class I-deficient β_2-microglobulin PHA blast splenocytes, no binding of anti-MUC1 Abs occurred (not shown).

We also noted that Abs VA2 (DTRPA), BCP8 (DTR), and BCP10 (RPAP) reacted with one of the HLA-A*0201 binding peptides (SAPDTRPAP) presented by HLA-A*0201-EBV-B cells (Table I), whereas BC2 (APDTR), VA1 (APG), and BCP9 (GSTAP; a control Ab) were nonreactive. In addition, HLA-A*0201-EBV-B cells pulsed with the second (high affinity) binding peptide (see below), HLA-A*0201 STAPPAHGV, were not detected by STAP31 (to amino acids AH); and control Ab BCP8 (DTR) was nonreactive (not shown). We lacked Abs to the N and C termini of the STAPPAHGV peptide, which would be appropriate, but of the Abs that were available, the pattern of reactivity as measured by flow cytometry was the same for HLA-A*0201 as for H2K^b and H2D^b class I molecules.

Blocking H2K^b MUC1 peptide and Ab binding by SIINFEKL, the high affinity H2K^b OVA peptide

As MUC1 peptides bind in the groove of class I molecules in a mode that can be detected by anti-MUC1 peptide Abs, it follows, therefore, that if MUC1 peptide binding was specifically blocked or inhibited by the binding of a high affinity competing peptide, then Ab binding should also not occur. As we had previously demonstrated that the OVA H2K^b binding peptide SIINFEKL competitively inhibited anti-MUC1 CTL lysis (11), RMA-S cells were incubated with SIINFEKL at increasing concentrations (0–20 μM) in the presence of a constant amount of MUC1 peptide (20 μM), and Ab binding was examined. In the presence of 5 μM SIIN FEKL, inhibition of Ab binding occurred with both VA2 (detecting the midpeptide epitope, . . . DTRPA.) and BCP10 (C-terminal epitope, ... RPAP) (Fig. 3B); increasing amounts of SIINFEKL (10–20 μM) completely inhibited Ab binding (Fig. 3, C and D). RMA-S cells in the presence of no peptide are shown in Figure 3A. The BC2 Ab (N-terminal epitope, APDTR . . .) again did not bind. These findings provide strong evidence that anti-MUC1 Abs bind to MUC1 peptide bound in the groove of class I molecules, but not when the peptide is displaced by a high affinity peptide.
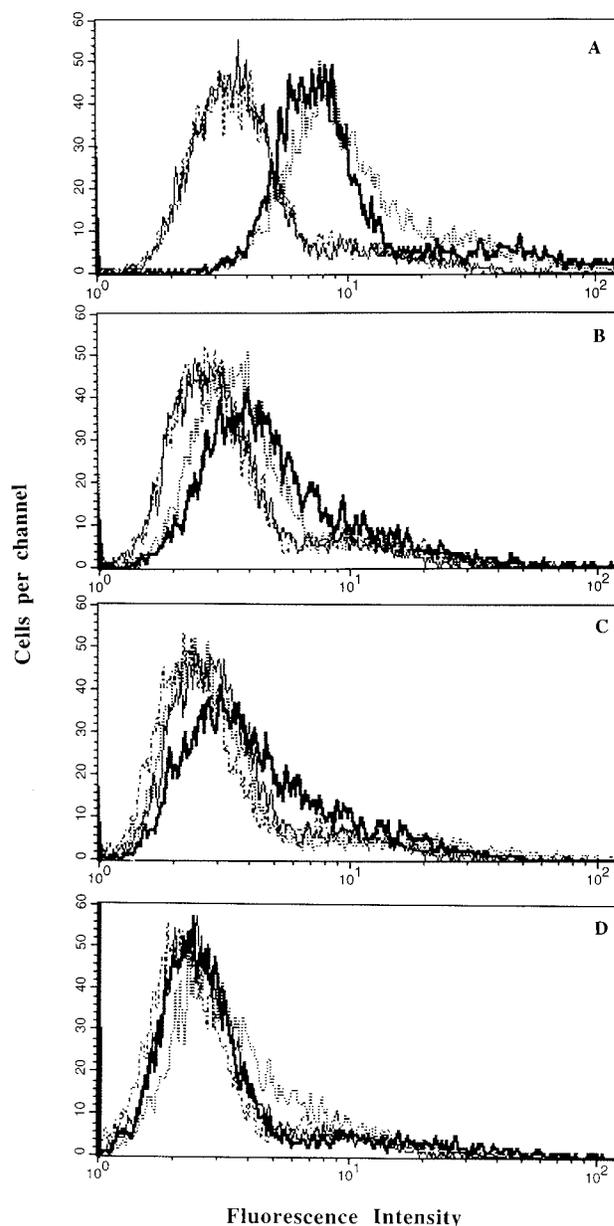

FIGURE 3. Flow cytometric analysis of RMA-S cells pulsed with 20 μM K^b-binding peptide (SAPDTRPAP) and increasing amounts of OVA peptide SIINFEKL (A, 0 μM; B, 5 μM; C, 10 μM; D, 20 μM) and incubated with anti-MUC1 peptide Abs; isotype control Ab (———), VA2 (····), BCP10 (———), BC2 (·—·—·). Ordinate, cells per channel; abscissa, fluorescence intensity.

Molecular modeling of H2K^b and H2D^b class I/MUC1 peptide interactions

Computer modeling was used to assess how the peptides SAP DTRPAP and APGSTAPPA could bind H2K^b and H2D^b, respectively, and which parts of the peptides could be exposed to Ab. The possible peptide conformers (from a putative side elevation if located in a class I molecule), are illustrated in Figure 4. For both peptides, it is seen that the general shape is bow-like, with the central residues (P4–P7) forming the apex of the bow. Interestingly, it is residues in this region that are recognized by the anti-MUC1 Abs and TCR molecules. Anti-MUC1 Abs recognize the motifs DTR, DTRPA, and RPAP (Fig. 1; Table I), further implicating D (P4) and T (P5) in recognition. For example, DT is known to be crucial for recognition by the BCP8 Ab, and Figure 4A shows
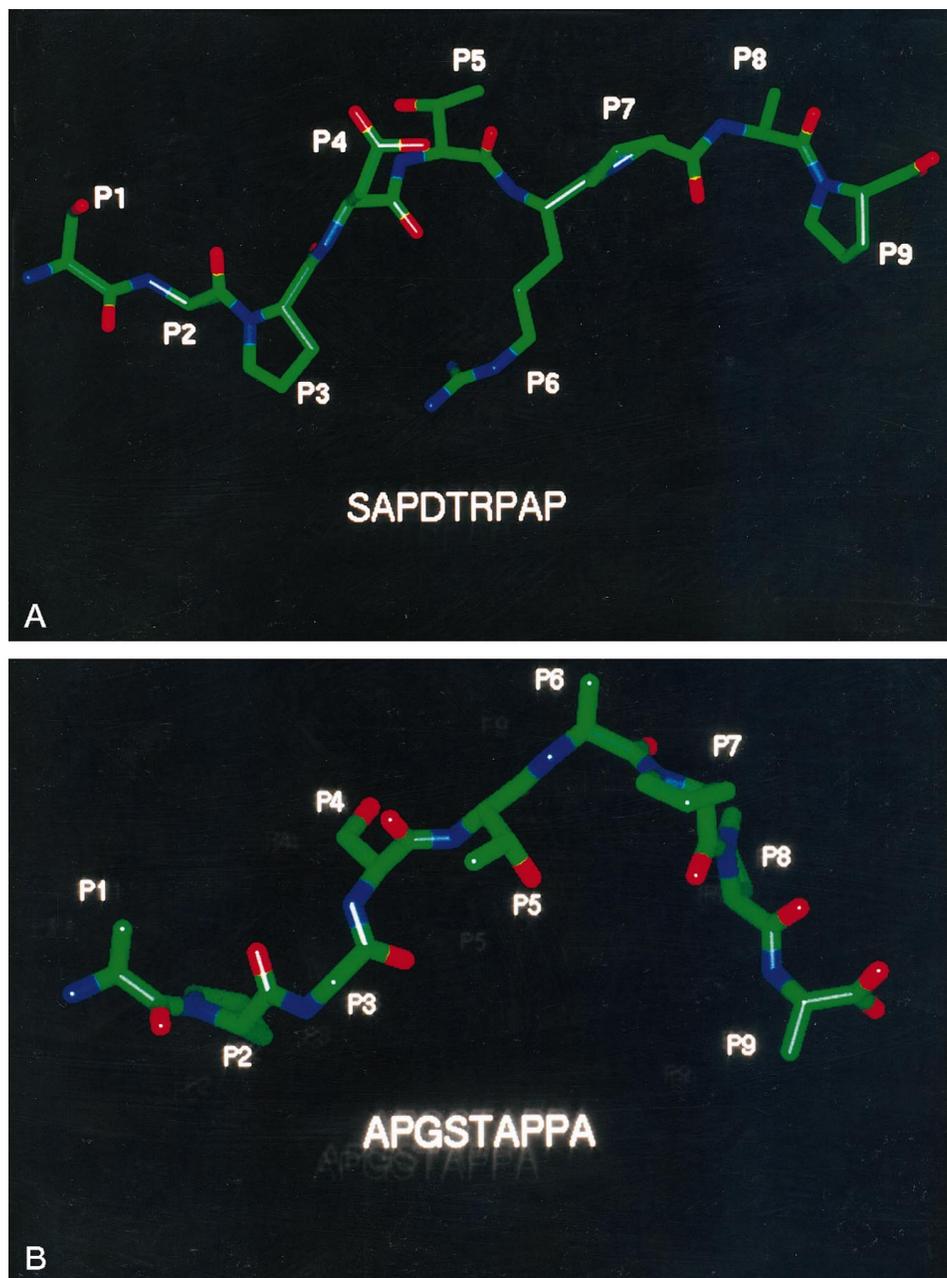
FIGURE 4. Computer model conformation of the H2K^b peptide SAPDTRPAP (*A*) and H2D^b peptide APGSTAPPA (*B*) viewed from the side when oriented for MHC class I binding. P1 corresponds to the N-terminal peptide residue. The colors are standard and represent N (blue), O (red), and C (green) atoms.

that these residues are upward facing, solvent exposed, and therefore accessible to Ab. In contrast, nearly all residues crucial for recognition by BC2 (APDTR) (25, 30) are buried (A and P at the N terminus; downward pointing R), which explains the lack of reaction of this Ab. Interestingly, in each of these motifs an R is present, yet in the model, R (P6) projects downward into the cleft, and Ab would not be available for detection. One possibility is that R is not important for recognition and that DT (P4P5) or PA (P7P8), also accessible to solvent, are sufficient. Alternatively, crystallographic studies of peptides binding to class I have shown that the central part of the peptide is very flexible, and a different orientation of R (P6) cannot be excluded. However, the model affords a third explanation, in that R155 of class I may be acting as a substitute for the R (P6). Mutational studies or use of the K^bm1 class I mutant (31) (K^bm1 has mutations at 152, 155, and 156) could be used to establish the orientation of the peptide. The SAPDTRPAP peptide is able to offer some stability to the H2K^b com-

plex (11), since it can be detected by CTLs. Figure 5*A* shows some of the interactions that may rationalize the stability and how the peptide might be oriented in the cleft. In the model, R (P6) forms a salt bridge to E24, as well as a hydrogen bond to N70 and S99, and thus acts as a good anchor residue for the peptide. Although Y and F are considered the standard anchor residues to bind to this part of H2K^b (7), other peptides, for example SRDHSRTPM (32), which lacks these residues, have been found to be high affinity binders for this class I molecule. The N terminus of the SAPDTRPAP sequence is supported by canonical hydrogen bonds to conserved residues Y159 and Y171. Notably, however, the C terminus of the SAPDTRPAP peptide does not appear to form any strong intermolecular links, suggesting poor binding to H2K^b in this region.

Modeling studies for APGSTAPPA suggested that S (P4), P (P7), and particularly A (P6) face upward and that T (P5) points downward (Fig. 4*B*). There is overall poor stability of the

FIGURE 5. The H2K[b]/SAPDTR PAP (*A*) and H2D[b]/APGSTAPPA (*B*) complexes viewed from above the Ag-binding cleft shown as the light green ribbon on the *top* and *bottom*. The class I residues shown (yellow) are those that are <2.5Å away from the peptide. Asp and Thr are identified in *A* and Ser, Ala, and Thr in *B*.
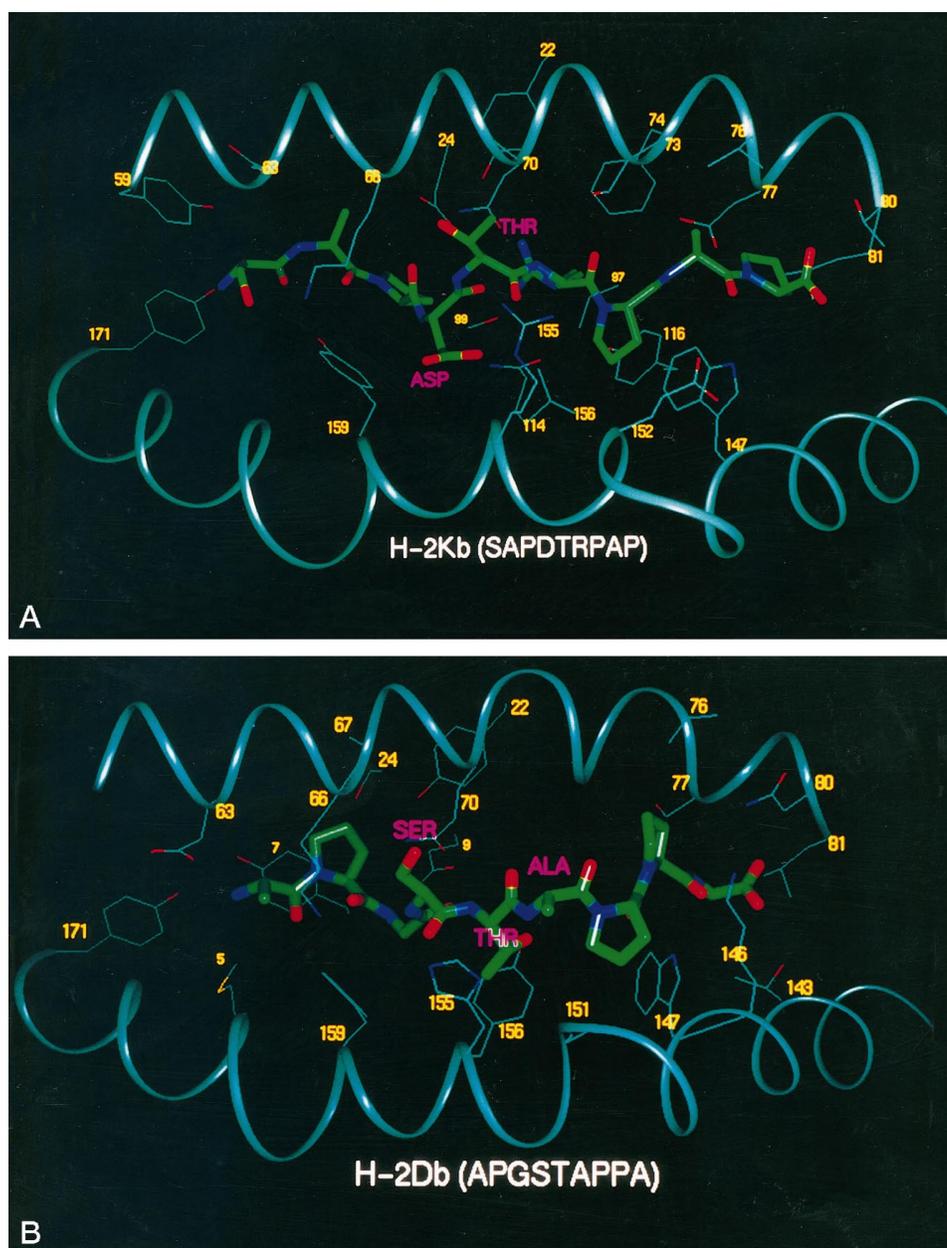
H2D[b]/APGSTAPPA complex, which can be attributed, in large part, to the lack of suitable anchor residues in the peptide (Fig. 5*B*). Indeed, a mutant peptide with T (P5) substituted by N (P5), an established anchor residue for H2D[b] peptides (7), introduces far more stability to the complex (11). The model (Figs. 4*B* and 5*B*) shows that although T (P5) reaches downward into the cleft, it is unable to form significant electrostatic interactions with H2D[b] because of the short side chain; with only one other polar residue S (P4), the peptide is also not able to form significant secondary anchors. It is in the central region (GSTAP; arching up) that the anti-MUC1 Ab BCP9 putatively might bind to the peptide in the class I groove. At the N terminus, however, canonical interactions bury the peptide so that the Ab VA1 (detecting APG) does not bind. The modeling studies for K[b] and D[b] with MUC1 peptides thus provide an explanation of how the Abs could selectively bind parts of the MUC1 peptides.

*Molecular modeling of HLA-A*0201 class I/MUC1 peptide interactions*

To investigate possible modes of HLA-A*0201 peptide binding, the peptides SAPDTRPAP and STAPPAHGV were computer modeled into the Ag-binding cleft of HLA-A*0201 (17). The models showed that the STAPPAHGV peptide, which contains a standard anchor residue (V) at P9, binds in the canonical way (not shown). This peptide is stabilized by hydrogen bonds involving residues Y7, Y159, and Y171 at the N terminus and D77, Y84, and W147 at the C terminus (not shown), as is usually the case in canonically bound peptides (12); K66 holds the N terminus down. By contrast, modeling indicated the significant deviation of the SAPDTRPAP peptide backbone as compared with STAPPAHGV (Fig. 6). At the N terminus, the SAPDTRPAP peptide is well anchored, forming six strong hydrogen bonds between MHC residues E63, Y99, Y159, and Y171 and peptide residues S (P1) and T (P2)

FIGURE 6. A comparison of the HLA-A*0201-modeled 9-mer (P1–P9) peptide conformations of the STAP PAHGV (green/red/blue) and SAP DTRPAP (magenta) peptides viewed from the side. The comparison is based on the superposition of the backbone of the Ag-binding domains (residues 10–170) of the complexes. The N terminus of the SAPDTRPAP peptide is deeper in the cleft and forms hydrogen bonds, while the C terminus is higher and is not bound in the conventional pockets (7). The STAP-PAHGV peptide is a canonical binding peptide.
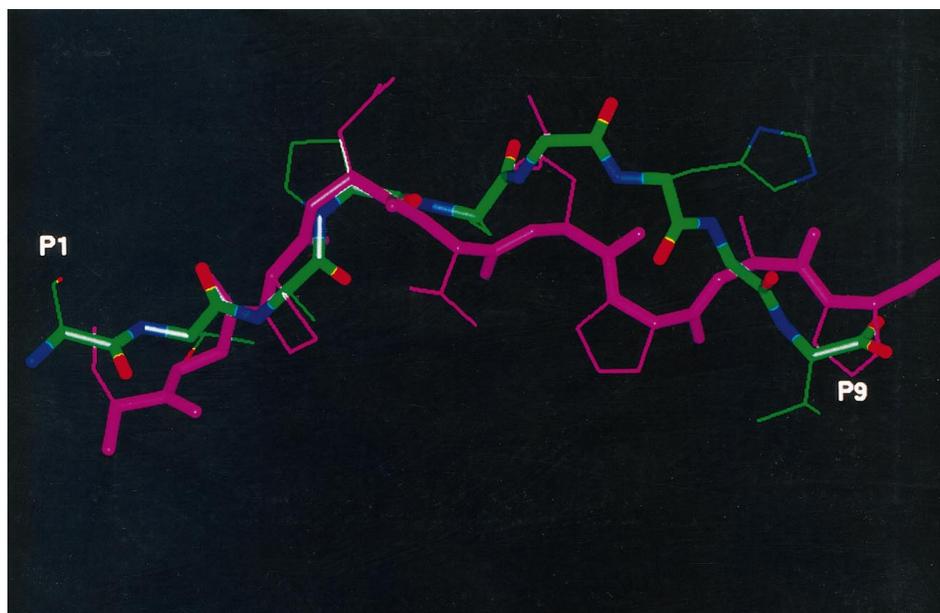
(not shown, Fig. 6). Thus, the modeling studies predict normal anchoring residues, which was confirmed by affinity studies in which the STAPPAHGV peptide bound with higher affinity than SAPDTRPAP (11). The complexed SAPDTRPAP peptide can be detected by VA2 (DTRPA) and BCP9 (DTR) Abs to the midregion and by BCP10 (RPAP) to the C terminus, but not by BC2 to the N terminus. A single hydrogen bond is likely to form between the backbone of P (P7) and T73, but there are no predicted interactions to other C-terminal peptide residues. Thus, the C terminus of the peptide is not well anchored in the groove and is predicted to loop out of the cleft, thereby exposed for Ab binding, with the peptide as a whole still anchored in the groove by the N terminus. There are several reasons for the poor fit of SAPDTRPAP to the HLA-A*0201 cleft. First, HLA-A*0201 has an R at position 97 at the base of the cleft; this large positively charged residue prevents R (P6) in the MUC1 peptide from acting as a secondary anchor by both steric and electrostatic effects. Also, the proline at the C terminus of the peptide prevents the formation of at least one peptide stabilizing hydrogen bond generally conserved in known peptide/MHC complexes. Further, P (P7), A (P8), and P (P9) are all nonpolar residues, and thus their side chains are unable to form hydrogen bonds or salt links with HLA-A*0201. A second model of the SAPDTRPAP-class I complex was also constructed (data not shown), orienting the peptide to project R (P6) down into the cleft. The resulting structure, after a conformational search with high temperature molecular dynamics, had a unique C-terminal structure in that the peptide did not reach to the end of the cleft, and a salt bridge was formed between R (P6) and the C terminus of the peptide. The N terminus, however, remained essentially canonical. Taken together, the models suggest reasons for poor binding of the C terminus of the SAPDTRPAP peptide and lend support to the notion that this part of the peptide may not be bound in the groove and is thus available for detection by the RPAP (BCP10) Ab. In addition, the modeling predicts that SAPDTRPAP would not bind as strongly as STAPPAHGV (because the former lacks the V anchor at P9), which has been confirmed by affinity studies (11, 29).

The molecular modeling is presented as evidence, but not proof of how peptides may be sitting in the class I groove and be accessible to Ab. It is accepted that modeling studies can be helpful as well as misleading and that direct evidence of the shape of the MUC1 peptides in the groove must come from crystallization stud-

ies, which are currently in progress. However the modeling was done under stringent conditions (see *Materials and Methods*), making use of existing crystal structures to form a template on which our MUC1 peptides were modeled, again not as good as crystals, but certainly a guide to possible confirmations.

Discussion

Crystallized structures of class I MHC and peptide molecule complexes allow an analysis of how some peptides bind within the groove of class I molecules (13, 14, 16, 17, 26, 32–34). A striking feature of these complexes is that the conformations of the peptide termini are similar, being stabilized by hydrogen bonds to the backbone of the peptide, involving class I molecule residues that are almost completely conserved despite the extensive class I polymorphism, e.g., for HLA the residues Y7, Y84, W147, Y159, and Y171. Peptide binding is facilitated by primary and secondary anchoring motifs, but with some central flexibility to allow TCR binding (15, 32, 35–37). Peptides that react in solid or liquid phase with Abs have previously been found to lose such reactivity when "buried" in the groove of the class I molecules; thus, Abs and T cells usually react with different epitopes, and anti-peptide Abs do not usually block T cell reactivity. The loss of Ab reactivity by peptides in the groove could be ascribed to changes in secondary structure, or more likely, it is due to amino acid residues being buried or obscured by proximity to the groove of class I molecules, particularly when there is high affinity binding. We previously reported that it was possible for antigenic peptides lacking recognized anchors to bind the MHC, albeit with low affinity, and to be recognized by CTLs (11). We now show that low affinity binding MUC1 peptides, complexed with class I molecules, can be accessible to anti-MUC1 peptide Abs. These findings are based on using mAbs that detect seven different epitopes in the MUC1 20-mer VNTR sequence.

The studies were performed in three ways: first, by blocking CTL effectors with anti-peptide Abs; second, by the detection of class I-bound peptide with Abs using flow cytometry; and third, by molecular modeling to explain how these peptides could bind to the class I groove and be accessible to both Abs and T cells. The findings are unusual, indeed to our knowledge are unique, for although rare Abs binding to class I and peptide complexed together

have been described (19, 20), in our study the Abs react only with the peptide. The ability to detect such peptides by Abs is likely to be due to three different reasons. First, we have a unique collection of Abs, which detect seven epitopes in the 20-amino acid MUC1 peptide and can detect different sites on the 9-mer peptides bound by class I molecules. Second, we can induce CTLs with ease by targeting the mannose receptor on macrophages (11, 21, 22, 28, 29). Third, the detection appears to result from the novel way in which low affinity MUC1 peptides bind in the groove, wherein the N terminus is buried, the middle portion arches upward to be free (and available for Ab detection), and with some peptides, the C terminus is also free and available to react with Ab. In addition to the peptides being bound in the MHC groove, which we assume to be the case because they are targets of T cells, there are two other possible interpretations to consider: 1) peptides binding to the cell surface separate from MHC molecules and 2) peptides binding to MHC class I molecules, but outside the groove. Regarding the first possibility, it is most unlikely that peptides bind to the cell surface in places other than with class I molecules, as the Abs did not detect peptides bound to cells of different haplotypes (22), and in the absence of class I molecules (in mice lacking β_2-microglobulin), there were no CTLs (22) and no reactivity with anti-MUC1 Abs. Thus, not only must class I molecules be present, they must be of the right haplotype to bind the peptide, and it is most unlikely that the peptides bind elsewhere than to class 1 molecules.

The second proposition, that peptides bind both inside the cleft (for detection by T cells) and on the class I molecule outside the cleft (which bind Ab), is also unlikely. The ability of anti-MUC1 peptide Abs to block CTL-mediated lysis is relevant, although such Abs could also block (by steric hindrance) if bound to peptides outside the groove. However, when competing peptides, e.g., SIINFEKL used in excess amounts (to prevent MUC1 peptide binding in the groove), then the Ab reactivity disappears (and anti-MUC1 CTLs no longer lyse cells), leading to the conclusion that binding occurs only in the groove (11). We were fortunate in having a collection of mAbs that react with sequential amino acids in the highly immunogenic APDTR region of the MUC1 VNTR sequence spanning peptides that can also be presented by class I molecules for recognition by CTLs. Some of the Abs were made by immunizing with breast cancer cell lines, others to purified mucins, yet others to synthetic peptides (23–25). The APDTR region, predictably, is the most immunogenic, and most of the anti-MUC1 peptide Abs characterized to date react within this region. The Abs detect sequential amino acids defined by using the "pin" technique, wherein peptides tethered at the C terminus can be used to map epitopes (23–25). Most of the MUC1 peptides can clearly and reproducibly be detected by these Abs in solid phase (e.g., ELISA), liquid phase, and on pins, and as these reactions are mostly independent of the secondary structure of the peptide, the conformation taken by the peptide in the class I groove would not be expected to alter their reactivity with Abs. However, why have such reactions not been found previously? Recently, there have been several descriptions of Abs recognizing peptide/class I interactions in which contiguous epitopes including both peptide and class I molecules were necessary for the reaction with Ab. Such descriptions include Abs bound to either OVA$_{257–264}$, vesicular stomatic virus NP$_{52–59}$, or influenza NP$_{345–360}$ peptides in complex with class I (19); other Abs have been shown to bind to viral peptides together with class I (38), to hen egg lysozyme peptide complexed with class II (39), or to influenza hemagglutinin (HA)$_{255–262}$ (20) (here, the peptide was of high affinity and only four amino acids in length was exposed to the TCR). Our peptides bound with a much lower affinity (11) and were likely to be less buried and hence more accessible to Ab. In our studies, it is ap-

parent that the same peptide sequence (SAPDTRPAP) can be presented by multiple H2 class I molecules (11). As in to the HIV1 permissive peptides of Berzovsky and colleagues (40), in whose study there is broad recognition of cytotoxic T cell epitopes from the HIV-1 envelope protein with multiple class I histocompatibility molecules, it is interesting to consider that all of these peptides could be exposed to bind not just to the TCR (perhaps in the absence of a direct MHC contribution) but also to Ab, as shown herein.

A second feature of importance in this study was the ability to easily generate CTLs to MUC1 peptides by targeting the mannose receptor (21, 22). Strangely, no CTL clones for MUC1 have been described, and their use would have aided this study. It is likely that the low affinity binding MUC1 peptides generate high avidity CTLs with high frequency (41), and in other studies, low affinity peptide-MHC interactions could lead to the induction of effective CTLs (42). We have since demonstrated that the H2K^b-, H2D^b-, and HLA-A*0201-binding MUC1 peptides bind with low affinity to class I; however the high avidity CTLs were generated (11, 29), in these cases the low affinity peptides lack defined anchor motifs. Since the MUC1 peptide binding to H2K^b, H2D^b, and HLA-A*0201 did not contain defined anchors, it is possible that the MUC1 peptides bind to the class I groove in an unusual manner, particularly as they are accessible to anti-peptide Abs (shown by flow cytometry) (Fig. 2; Table I) and by the ability of anti-MUC1 Abs to block CTLs (Fig. 1; Table I). The Ab reaction with the H2K^b- or HLA-A*0201-binding peptide (SAPDTRPAP) is likely to occur with a loop (e.g., DTR) and to the free C-terminal end; the N terminus is buried and not accessible to Abs, all of which are features predicted by the computer-generated models (Figs. 3–5). The Ab binding to APGSTAPPA in H2D^b is likely to occur in the GSTAP loop; the N and C termini of the peptide and not being accessible to Abs. Similarly, an Ab to the -AH- epitope was nonreactive with the canonical HLA-A*0201-binding peptide, STAP PAHGV. The experimental observations of Ab binding and the structural modeling were completely consistent. Although 8- to 9-amino acid peptides are required to make full contact and sit snugly in the class I groove, it is not unusual for loops to be formed in the peptide backbone. Indeed, loops have been described using peptides longer than 9 amino acids, when the bulging of the peptide backbone is more prominent (17) as it loses hydrogen bonding interactions with the MHC in the central peptide region. For example, a peptide that has anchoring amino acids for H2K^b (Y at P5 and L at P8) is the Sendai virus NP$_{324–332}$ (FAPGNYPAL), which forms a kink at P4 and P5 causing these amino acids to make few contacts with the residues of the class I cleft (13). A similar observation was also noted for HLA-Aw68 and the influenza flu NP peptide (KTGGPIYKR), which kinks out from the cleft at P3 and P4, resulting in no hydrogen bonds with residues of the cleft for P4–P7 (34). It should also be noted that there are prolines present in these two peptides that disrupt hydrogen bonding of the peptide to the class I cleft (43). The MUC1 H2K^b/HLA-A*0201 (SAP DTRPAP) and H2D^b (APGSTAPPA) peptides are rich in prolines, which may assist in the MUC1 peptides looping out from the class I cleft to expose the central region or the C terminus. These issues could be investigated by using the panel of K^{bm} class I mutant mouse strains, in which the effects of particular class I amino acid substitutions on peptide binding, accessibility to Ab, and effects on T cell recognition could be evaluated. In a recent study, peptides eluted from HLA class I molecules were found to lack the consensus C-terminal hydrophobic or positively charged amino acids (44); it would be of interest to see whether the C termini of these peptides are solvent exposed and also accessible to anti-peptide Abs.

In conclusion, it is clear that anti-MUC1 peptide Abs can react with specific peptides in the groove of class I molecules, shown by blocking CTLs and directly by flow cytometry with anti-MUC1 Abs. Computer-generated models provided a structural basis for interpretation of the data. The findings are unique, no doubt due to the availability of Abs detecting sequential MUC1 epitopes, the ease of generation of CTLs by mannose receptor targeting, and the unique way in which MUC1 peptides of low affinity are likely to bind in the class I groove. Modeling studies suggested that although the N terminus was buried (and inaccessible), the middle region was accessible to MUC1 Abs as was, at times, some poorly anchored C termini. The findings demonstrate that not all peptides need to be firmly anchored to be detected by CTLs and that the MUC1 peptide Ab system could be useful in detecting peptide-class I interactions.

Acknowledgments

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