Apparent Lack of MHC Restriction in Binding of Class I HLA Molecules to Solid-Phase Peptides

By Benjamin P. Chen, Jonathan Rothbard,* and Peter Parham

From the Department of Cell Biology, Stanford University, Stanford, California 94305; and *ImmuLogic Pharmaceutical Co., Palo Alto, California 94304

Summary
The specificity of binding of solubilized, purified HLA-A,B molecules to solid-phase peptides has been examined using the assay described by Bouillet et al. [1989. Nature (Lond.) 339:473.] 64 peptides derived from the sequences of viral antigens, HLA-A,B,C heavy chains, and clathrin light chains were tested for binding to HLA-A2.1, Aw68.1, Aw69, B44, and B5, molecules that differ by 5-17 residues of the peptide binding groove. 15 of the peptides, including those known to be T cell epitopes, gave significant binding. The pattern of peptide binding for each of the five HLA-A,B molecules was not significantly different. Binding was demonstrated to be a property of native β2m-associated HLA-A,B molecules that preserved conformational antigenic determinants after binding to peptide. In comparison to our previous results from solution-based assays the proportion of HLA-A,B molecules that can bind solid-phase peptides is very high. This accessibility of solid-phase peptides to the binding site of MHC molecules may be directly related to the observed absence of MHC specificity in the binding.

Materials and Methods

Peptides. Peptides were synthesized by a solid-phase method using Fmoc or Boc chemistry. Homogeneity of the peptides was indicated by reverse-phase HPLC.

Isolation of HLA Molecules. HLA molecules were purified from detergent solubilized cell lysates by immunoaffinity chromatography as previously described (13). HLA-A2.1, Aw69, and Aw68.1 molecules were purified from cell lysates of human lymphoblastoid...
B cell lines 721.53 (A2), IDF (Aw69, A26, B18, Bw38), and CIR.Aw68.1 (a HLA-A,B-negative cell line transfected with the Aw68.1 gene), respectively, using a monoclonal antiA2, Aw69, Aw68 (CR11.351) antibody column. HLA-B5 and B44 molecules were purified from cell lysates of B-lymphoblastoid cell line 721.144 (B5) and C1R.B44 (a B44 transfectant), respectively, using a monomorphic anti-HLA-A,B,C (W6/32) monoclonal antibody column.

Peptide Binding Assay.

Direct binding of radiolabeled HLA molecules to peptides precoated on polystyrene microtiter wells was as described by Bouillot et al. (12). Briefly, HLA molecules were labeled with 1 mCi of 125I (Amersham Corp., Arlington Heights, IL) by the chloramine T method and fractions containing radiolabeled HLA molecules were collected from a Sephadex G50 column. The HLA molecules were diluted in PBS containing 0.5% (NP-40), 1% BSA, 0.02% NaN₃, and 1 mM PMSF. Peptides at 1 mg/ml were plated in duplicates in polystyrene microwell plates. Plates containing 10¹⁴1 peptides per well were incubated for 16 h at room temperature and washed twice with 0.1% BSA in PBS. Unoccupied sites were saturated with 1% BSA in PBS for 1 h. The plates were washed three times and radiolabeled HLA molecules were added and placed at room temperature for 18 h. The plates were then washed three times and radioactivity bound per well was measured by direct counting in a gamma counter.

Detection of HLA by mAbs.

HLA molecules bound on peptide-precoated plates were detected by anti-HLA mAbs and radiolabeled rabbit anti–mouse antibodies as secondary and tertiary reagents. HLA molecules were added to peptide precoated plates for 18 h at room temperature then washed three times with 0.1% BSA in PBS followed by incubation with 1% ovalbumin in PBS for 30 min.

Figure 1. Primary amino acid sequences of peptides. Peptides were synthesized on a Milligen/Biosearch 9600 automatic peptide synthesizer using Fmoc chemistry. β₂m = β₂ microglobulin; Flu = influenza; M = matrix protein; NP = nucleoprotein; HIV = human immunodeficiency virus; LMP = latent membrane protein of Epstein-Barr virus; LCα, LCβ = light chain α and β of clathrin proteins.

Table 1

| Peptide Number | Sequence                        | Binding Assay | Detection of HLA by mAbs |
|---------------|--------------------------------|---------------|-------------------------|
| 1             | A1 60-84 WDGETRMKANSQTRQDLRGLGY |               |                         |
| 2             | A2 98-113 MYGCDVGSDQRFLRG       |               |                         |
| 3             | A2 56-69 GPEYWDGETRKVKA         |               |                         |
| 4             | A2 57-69 -PEFWDGETRKVKA         |               |                         |
| 5             | A2 58-69 --EYWDGETRKVKA         |               |                         |
| 6             | A2 59-69 --YWGDGETRKVKA         |               |                         |
| 7             | A2 60-69 --YWGDGETRKVKA         |               |                         |
| 8             | A2 61-69 -----GETRKVKA          |               |                         |
| 9             | A2 62-69 -----GETRKVKA          |               |                         |
| 10            | A2 60-84 WDGETRKVKAHSQTRQDLRGLGY |               |                         |
| 11            | A2 70-85 HSQTRQDLRGLGY          |               |                         |
| 12            | A2 87-102 QSEAGSHTVQRMGCD       |               |                         |
| 13            | A2 112-129 KYDIALKEDLSWTA       |               |                         |
| 14            | A2 141-160 QTTHKREAASQQRFLRKL  |               |                         |
| 15            | A2 161-171 GTCEVWLQRY           |               |                         |
| 16            | A2 146-161 KWSAAARASQQRFLRKL   |               |                         |
| 17            | A2 137-148 DMAQTQHKHE           |               |                         |
| 18            | A2 266-282 LPMFLT2WEPSSQTTI     |               |                         |
| 19            | Aw24 60-84 WDGETRQKANSQTRQDLRGLGY |         |                         |
| 20            | Aw24 70-85 HSQTRQDLRGLGY        |               |                         |
| 21            | Aw68.1 141-160 QTTHKREAASQQRFLRKL |         |                         |
| 22            | Aw68.1 98-113 MYGCDVGSDQRFLRG  |               |                         |
| 23            | Aw68.1 60-84 WDHRNTRVKAQASQTRQDLRGLGY |       |                         |
| 24            | Aw68.1 56-69 GPEYWDGETRKVKA     |               |                         |
| 25            | Aw68.1 60-84 WDRETQKANSQTRQDLRGLGY |         |                         |
| 26            | Aw68.1 61-69 -----GETRKVKA      |               |                         |
| 27            | Aw68.1 62-69 -----GETRKVKA      |               |                         |
| 28            | Bw58 56-69 GPEYWDGETRKVKA       |               |                         |
| 29            | Bw58 60-84 WDGETRNNKASKATQYTRNLRALRY |         |                         |
| 30            | B7 60-84 WDHRNTRVKAQASQTRQDLRGLGY |         |                         |
| 31            | Bw27.1 60-84 WDGETRQKANSQTRQDLRGLGY |        |                         |
| 32            | Bw27.2 60-84 WDGETRQKANSQTRQDLRGLGY |        |                         |
| 33            | B13 60-84 WDRRETQKANSQTRQDLRGLGY |         |                         |
| 34            | B14 60-84 WDRRETQKANSQTRQDLRGLGY |         |                         |
| 35            | B41 60-84 WDRRETQKANSQTRQDLRGLGY |         |                         |
| 36            | B42 60-84 WDRRETQKANSQTRQDLRGLGY |         |                         |
| 37            | B44.1 60-84 WDRRETQKANSQTRQDLRGLGY |        |                         |
| 38            | B44.1 141-160 QDRKREAASQQRFLRKL |         |                         |
| 39            | B47 60-84 WDRRETQKANSQTRQDLRGLGY |         |                         |
| 40            | Cw1 60-84 WDRRETQKANSQTRQDLRGLGY |         |                         |
| 41            | Cw2.1 60-84 WDRRETQKANSQTRQDLRGLGY |        |                         |
| 42            | Cw3 60-84 WDRRETQKANSQTRQDLRGLGY |         |                         |
| 43            | B₃m 1-16 IQRTQPKFYSSRHKPA       |               |                         |
| 44            | B₃m 8-25 QYRSHPRAEWKSFLH      |               |                         |
| 45            | B₃m 25-41 CVYSGFHSOIDEVDLEK    |               |                         |
| 46            | B₃m 38-54 DLLNKRKREKVEHSD      |               |                         |
| 47            | B₃m 51-66 HSDFLFSKWSFELY       |               |                         |
| 48            | B₃m 67-79 YETTTEPKDEYQA        |               |                         |
| 49            | B₃m 80-94 CRVWHTLSQPKVXK       |               |                         |
| 50            | B₃m 83-99 NHVTSQPKIVWDRMD      |               |                         |
| 51            | FLU M57-68 KGLGTYFTLV         |               |                         |
| 52            | LMF VM6QDWTGLALL               |               |                         |
| 53            | HIV.gag 265-279 KRWILGKIVWRY   |               |                         |
| 54            | FLU.MP335-349 SFAFLRLWISPFG    |               |                         |
| 55            | LCa 47-71 FADIGGAPGPFHHEPPOGPDVOD |       |                         |
| 56            | LCa 113-137 MEREADNASKFSAKEMEKAIKE |       |                         |
| 57            | LCa 148-164 QLRKRTANKWAWAAEAFV |         |                         |
| 58            | LCa 188-208 AAXEARFVNGIEEESPFTEDER |       |                         |
| 59            | LCa 204-226 YHFRVSDCDFNFKSSQKAXKVDV |       |                         |
| 60            | LCb 45-72 ECGPAGRASHAPQPGTSGSEGONG |       |                         |
| 61            | LCb 113-135 RKRQELDAAKVEQDMDEAK |         |                         |
| 62            | LCb 148-164 QVRERINNRAEAFV    |               |                         |
| 63            | LCb 188-208 AASAEARFVNGIEEESPFTEDER |       |                         |
| 64            | LCb 208-229 KVAQCDFNPFSKSSQKCOVRL |       |                         |
at 4°C. After incubation, the microwells were washed twice and anti-HLA mAbs (250 μg/ml) were added to each well and placed at 4°C for 45 min. At that time, the microwells were then washed three times with 0.1% BSA in PBS and radiiodinated rabbit anti-mouse F(ab')2 were added to each well at 600,000 cpm/well. The plates were placed at 4°C for 45 min, then washed three times and radioactivity bound per well was counted.

Results

Comparison of the crystallographic structures of HLA-A2.1 and HLA-Aw68.1 revealed differences in the detailed architecture of the peptide binding groove, indicating that these molecules would exhibit distinctive peptide binding specificity (5). In an attempt to identify discriminating peptides we tested 64 peptides for binding to HLA-A2.1 and HLA-Aw68.1 using the solid-phase assay of Bouillot et al. (12). Binding to HLA-Aw69, which has a hybrid peptide binding groove (α1 derived from HLA-Aw68.1 and α2 derived from HLA-A2.1) and to HLA-B44 and HLA-B5 was also assessed. The peptides tested had sequences derived from various viral antigens, HLA-A,B,C heavy and light chains and clathrin light chains (Fig. 1).
Fifteen of the 64 peptides bound to HLA-A2.1 at levels >10-fold of background, 15 peptides gave binding twofold above background levels and 34 were negative (Fig. 2 A). Positively binding peptides were found among the HLA peptides and the viral peptides, but not among the clathrin peptides. The lack of HLA binding to the clathrin peptides was not due to poor absorption of peptides to the microtiter plates as many of these peptides bind to specific anti-clathrin monoclonal and polyclonal antibodies in similarly configured assays (14). Thus, there is, as previously demonstrated (12), discrimination between peptides in this assay. Calculation also shows that up to 30% of the total input of radio-iodinated HLA-A2.1 molecules can bind to solid-phase peptide. Among the strongly binding peptides is that corresponding to residues 57–68 of the influenza matrix peptide (Flu.M57-68) and the binding of HLA-A2.1 to this peptide we observed is comparable to that reported by Bouillot et al. (12). This peptide has repeatedly been shown to be presented to influenza specific CTL by HLA-A2.1 and HLA-Aw69 (15). In addition, Gotch et al. (16) have tested peptide analogues for their capacity to substitute for Flu.M57-68 and have identified positions at which substitution prevents presentation to specific CTL. The pattern of binding of HLA-A2.1 to these analogue peptides observed in the solid-phase assay closely correlates with the pattern of sensitization observed by Gotch and colleagues (Gotch, F., and McMichael, A. J., unpublished data, personal communication), showing that among this highly related set of peptides, binding correlates with presentation (Fig. 2 B) with the exception that peptides E63, Y63, and F63 bound in the solid-phase assay and were not recognized by CTL and did not inhibit presentation of the matrix peptide by HLA-A2.

It was therefore surprising that no significant discrimination between five different MHC molecules was observed in this binding assay; the pattern of binding of HLA-A2.1 to the 64 peptides was mimicked by HLA-Aw68.1, Aw69, B5, and B44 (Fig. 2). Thus every peptide that bound to one HLA molecule bound to the other four; peptides that failed to bind to one HLA molecule did not bind to the other four. Even when the levels of binding of different peptides to the five HLA molecules were compared, there were no significant differences that could be ascribed to “HLA type.”

These results clearly challenge the hypothesis that variation between class I MHC molecules, which is mainly found in the peptide binding groove, acts significantly to alter the peptide binding specificity. The five HLA-A,B molecules studied here show considerable variation in the residues that point into the groove, between 5 and 17 amino acid differences (Fig. 3), and yet their pattern of peptide binding appears identical.

This raised the possibility that it was denatured HLA molecules, or an iodinated contaminant in the HLA preparations, that was binding the peptides. To address this question we separated radio-iodinated HLA-A2 on a column of a W6/32, a mAb that only binds to native β2m-associated molecules, and measured the binding to peptides of the bound and the unbound radioactivity. All peptide binding activity of the 125I-HLA preparation was removed by passage over the W6/32 column, showing that the observed peptide binding was due to β2m-associated HLA-A2 heavy chains and not to free heavy chains or a contaminant. This conclusion was confirmed by the recovery of peptide binding activity by elution of material bound to the W6/32 column. Moreover,
Table 1. Binding of Peptides by Radioiodinated HLA-A2.1 Molecules Reisolated by Affinity Chromatography

| Peptides       | Pre W6/32 column | Eluate | Run through |
|----------------|-----------------|--------|-------------|
| FLU.M57-68    | 600,000*        | 50,000 | 50,000      |
| Bw58.60-84    | 7,640†          | 1,234  | 6,585       | 638          |
| B41.60-84     | 11,347          | 1,876  | 13,856      | 1,214        |
| None          | 1,812           | 1,180  | 1,593       | 440          |

* Amount of radioactivity added to each well.
† Amount of radioactivity bound to each well after three washes to remove excess radioactivity.

only a fraction (1/10) of the radioactivity was needed to achieve binding comparable to the level obtained with unfractionated radiolabeled HLA-A2 (Table 1).

A further source of potential artefact is modification of the HLA molecules by the iodination reaction. The peptide binding groove of MHC molecules is characterized by a number of strategically placed tyrosine residues (4) and it is possible that their iodination could alter the combining site specificity to give a degenerate pattern of peptide binding.

To circumvent this issue we developed a modified assay in which the binding of noniodinated HLA molecules to plate-bound peptides was measured using specific anti-HLA-A,B mouse mAbs and radioiodinated anti-mouse Ig (Fig. 4). This indirect assay gave similar patterns of peptide binding to those seen in the direct assay and equivalent results were obtained with six mAbs directed against different epitopes of HLA-A,B molecules; results obtained with W6/32 and A2.1 mAb are shown (Fig. 4 A). Protein modification due to radio-iodination is therefore not the source of the degenerate binding.

Discussion

Bouillet et al. (12) previously analyzed the binding of radioiodinated HLA-A2.1, HLA-B37, and HLA-B27 to solid-phase peptides. Peptides that were known to be antigenic for T cells bound to their appropriate restriction element, but although some quantitative differences were observed, these authors found that peptides binding to one class I molecule tended to bind to others. In three cases where direct comparison can be made, the binding of HLA-A2.1 to influenza nucleic protein peptide FLU.NP335-349, influenza matrix protein peptide FLU.M57-68, and HIV gag protein peptide HIV.gag.265-279, we obtain comparable results to Bouillot et al. Furthermore we find the assay robust, highly reproducible, and suitable for screening large numbers of samples.

Comparison of the binding of the same MHC molecule to different peptides showed specificity that correlated with
presentation to T cells. In contrast we found no significant differences in the patterns of peptide binding of five different class I molecules. Bouillot et al. were able to discern some quantitative differences in titration experiments. In this study peptides were plated at 1 mg/ml, a concentration that for known antigens gave maximal binding, and this protocol may have served to reduce the discriminating capacity of the assay. Nevertheless it is clear from our results and those of Frelinger et al. (17) with this assay that it is hard to argue for a profound effect of MHC polymorphism on the binding of peptides to HLA-A,B molecules. The critical issue is whether this is a true reflection of the specificity of peptide binding by class I molecules or an artefact of the assay.

Trivial artefacts have been ruled out. The binding is clearly the product of βm-associated HLA-A,B heavy chains and is not dependent upon radioiodination. Conformational antigenic determinants of the HLA-A,B molecules are preserved on binding to solid-phase peptides. In addition we know that binding does not require the presence of detergent or the hydrophobic transmembrane region of the HLA-A,B molecule. Papain solubilized and genetically engineered, secreted HLA molecules give similar binding to the detergent solubilized product.

These results combined with the peptide specificity and its correlation with T cell reactivity argue for binding being within the peptide binding groove. That the binding grooves of a much greater proportion of HLA-A,B molecules are accessible to solid-phase peptides than to peptides in solution suggests that interactions at the solid phase facilitate either displacement of the endogenous peptide or the simultaneous binding of the endogenous peptide and the experimentally offered peptide. This might involve nonphysiological interactions between the class I molecules and plastic that loosen up the structure and thereby reduce the specificity of the combining site. Alternatively the plastic surface might mimic conditions that MHC molecules encounter in vivo and which contribute to their function.

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Address correspondence to Dr. P. Parham, Department of Cell Biology, Sherman Fairchild Building, Stanford University, Stanford, CA 94305.

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