HLA Photoaffinity Labeling Reveals Overlapping Binding of Homologous Melanoma-associated Gene Peptides by HLA-A1, HLA-A29, and HLA-B44

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Immanuel F. Luescher‡§, Pedro Romero‡, Dmitry Kuznetsov‡, Donata Rimoldi‡, Pierre Coulie¶, Jean-Charles Cerottini‡, and C. Victor Jongeneel‡

From the Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, 1066 Epalinges, Switzerland and the Ludwig Institute for Cancer Research, Brussels Branch, 1200 Brussels, Belgium

Melanoma-associated genes (MAGEs) encode tumor-specific antigens that can be recognized by CD8+ cytotoxic T lymphocytes. To investigate the interaction of the HLA-A1-restricted MAGE-1 peptide 161–169 (EADPTGHSY) with HLA class I molecules, photoreactive derivatives were prepared by single amino acid substitution with N-[(iodo-4-azidosalicyloyl)L-2,3-diaminopropionic acid. These derivatives were tested for their ability to bind to, and to photoaffinity-label, HLA-A1 on C1R.A1 cells. Only the derivatives containing the photoreactive amino acid in position 1 or 7 fulfilled both criteria. Testing the former derivative on 14 lymphoid cell lines expressing over 44 different HLA class I molecules indicated that it efficiently photoaffinity-labeled not only HLA-A1, but possibly also HLA-A29 and HLA-B44. MAGE peptide binding by HLA-A29 and HLA-B44 was confirmed by photoaffinity labeling with photoreactive MAGE-3 peptide derivatives on C1R.A29 and C1R.B44 cells, respectively. The different photoaffinity labeling systems were used to assess the ability of the homologous peptides derived from MAGE-1, -2, -3, -4a, -4b, -6, and -12 to bind to HLA-A1, HLA-A29, and HLA-B44. All but the MAGE-2 and MAGE-12 nonapeptides efficiently inhibited photoaffinity labeling of HLA-A1, which is in agreement with the known HLA-A1 peptide-binding motif (glutamic acid in P3 and C-terminal tyrosine). In contrast, photoaffinity labeling of HLA-A29 was efficiently inhibited by these as well as by the MAGE-3 and MAGE-6 nonapeptides. Finally, the HLA-B44 photoaffinity labeling, unlike the HLA-A1 and HLA-A29 labeling, was inhibited more efficiently by the corresponding MAGE decapeptides, which is consistent with the reported HLA-B44 peptide-binding motif (glutamic acid in P2, and C-terminal tyrosine or phenylalanine). The overlapping binding of homologous MAGE peptides by HLA-A1, A29, and B44 is based on different binding principles and may have implications for immunotherapy of MAGE-positive tumors.

MAGE1 is a family of at least 12 related genes that are expressed in tumors of various histological types but not in normal adult tissue, except for testis (1). The identification of MAGEs and CTL epitopes they encode relied on the use of melanoma cell lines andalogous MAGE-specific CTL clones (1–4). The first MAGE CTL epitopes that have been mapped are the HLA-A1-restricted MAGE-1 peptide 161–169 (EADPTGHSY) and the homologous MAGE-3 peptide 168–176 (EVDPIGHLY) (4–6). Other MAGEs encode related sequences, which, except for MAGE-2 and MAGE-12, also contain the known HLA-A1 peptide-binding motif, e.g. an acidic residue in position 3 and a C-terminal tyrosine (4, 7–9).

Since the availability of MAGE-specific CTL clones is limited and the identification of epitopes they recognize is cumbersome, it is likely that there exist several as yet unidentified MAGE CTL epitopes or the MAGE CTL epitopes could be presented by other HLA molecules. The latter possibility is suggested by recent reports showing that some groups of HLA class I molecules overlap in peptide binding specificity (10–12).

In the present study we utilized HLA class I photoaffinity labeling to investigate whether HLA-A1 binding MAGE peptides can also bind to other HLA molecules. We have previously shown that the interaction of antigenic peptides with MHC class I molecules can be assessed on cells by photoaffinity labeling (13–15). In the systems studied so far, MHC class I photoaffinity labeling was remarkably allele-specific. Moreover, the lack of significant labeling of other cellular components made possible the analysis of photoaffinity labeling by direct SDS-PAGE of lysates of labeled cells (13–15). The main limitation of this approach was the synthesis and identification of suitable photoreactive and radiolabeled peptide derivatives. To overcome this difficulty we have recently introduced a novel synthesis strategy that permits the preparation of such peptide derivatives by automated solid phase peptide synthesis in which single amino acids are replaced with photoreactive N-[(iodo-4-azidosalicyloyl)L-2,3-diaminopropionic acid (Dap(IASA)) (15).

Here we show that suitable HLA binding photoreactive derivatives of MAGE-1 and -3 peptides can be identified by systematic testing of single Dap(IASA)-substituted peptides. Photoaffinity labeling experiments revealed that the MAGE-3 peptides 168–176 and 167–176, as well as homologous MAGE peptides, can bind not only to HLA-A1 but also to HLA-A29 and HLA-B44. This photoaffinity labeling approach, combined with molecular modeling, constitutes a straightforward means to identify overlapping peptide binding by HLA class I molecules.
Photoaffinity Labeling of HLA Class I Molecules

MATERIALS AND METHODS

Cell Lines and Transfectants—Most of the cell lines were generous gifts from different laboratories (as described under “Acknowledgments”). The homologous EBV transformed lymphoblastoid cell lines were described at the 10th International HLA Workshop (16, 17). The C1R transfectants were prepared by transfecting C1R cells, which express no significant levels of HLA-A and -B proteins and very low levels of HLA-Cw4 (18), with expression vectors encoding HLA-A1, -A29, or -B4403 essentially as described (29). COS-7 cells were transiently transfected with a vector encoding HLA-Cw*1601 using lipofectin (Life Technologies, Inc.) and were used 24 h after transfection. All cells were cultured in RPMI 1640 medium supplemented with 1-glutamine and 5% fetal calf serum.

Peptide and Conjugate Synthesis—Reagents for peptide and conjugate synthesis were obtained from Novabiochem (Lucerne, Switzerland), Bachem Finechemicals (Bubendorf, Switzerland), and Fluka (Buchs, Switzerland). General reagents were from Sigma (Chemie, Buchs, Switzerland). All synthetic procedures were performed essentially as described previously (15). In brief, the photoreactive MAGE-1 peptide derivatives were prepared in two steps. In the first step the peptide derivatives containing Dap(ASA)-ADPTGHSY(PO3H2) were synthesized by conventional solid phase peptide synthesis, based on the Fmoc (N-(9-fluorenylethyl)loxycarbonyl) strategy. In the second step these derivatives were subjected to chloramine T iodination with 125I or nonradioactive iodine and dephosphorylated with alkaline phosphatase (15). Upon elution with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid, rising within 1 h from 0 to 75%, the derivative MAGE-1 E161Dap(ASA) (E161X) eluted after 27.2 min, A162X after 26.9 min, P164X after 26.5 min, T165X after 28.4 min, G166X after 26.4 min, H167X after 28.7 min, and S168X after 28.4 min. Dap(ASA) derivatives of the MAGE-3 peptides 166–178 and 167–176 (MEVD-PICHGLY) were prepared likewise. While the decapeptide derivatives eluted only slightly later (~1 min) from the HPLC column than the corresponding nonapeptide derivatives, they all eluted considerably later than the homologous MAGE-1 peptide derivative. The MAGE-3 decapeptide derivative M167X eluted after 40.1 min, V169X after 35.7 min, D170X after 40.5 min, P171X after 36 min, I172X after 34.5 min, G173X after 36.4 min, H174X after 39.4 min, and L175X after 35.2 min. All peptides and conjugates were analyzed by mass spectrometry on a LDI 1700 mass spectrometer (Linear Scientific Inc., Reno, NV) and by UV spectrometry on an in-line 1000S diode array UV spectrometer (ABI, Foster City, CA). All Dap(ASA) peptide derivatives displayed the expected UV absorption maxima at 214, 270, and 310 nm and after iodination at 214, 272, and 325 nm. The observed M, corresponded with the calculated M, but in most cases a second M was also observed. The mass difference of approximately 2000Ci/mmol, and their aqueous solutions of 0.7–1.1 × 106 cpm/μl were used within 1 week.

Photoaffinity Labeling—All labeling procedures were performed essentially as described previously (13–15). In brief, cells (6 × 106/ml) were resuspended in RPMI 1640, supplemented with HEPES (10 mm) and 10% dialyzed fetal bovine serum (Whittaker, Walkersville, MD), and incubated in 1-ml aliquots with the iodinated peptide derivative (2 × 107 cpm/ml) and human β2-microglobulin (2.5 μg/ml; Sigma) in 6-well plates (Costar) at 26 °C for 3–4 h. Photolabeling was induced by UV irradiation at 254 nm with a 15-W mercury lamp; the fluorescence yield at 365 ± 40 nm (Bioblock, Illkirch, France). After the addition of protease inhibitors (leupeptin, iodoacetamide, and PMSF) and HEPES (50 mm) the cells were lysed at 0–4 °C with Nonidet P-40 (0.7% final concentration), and the detergent-soluble fractions were subjected to immunoprecipitation with W6/32 mAb (22). Alternatively, the UV-irradiated cells were washed 3 times with 15 ml of Dulbecco’s modified Eagle’s medium containing 2% fetal calf serum and 1 time with phos phosphate-buffered saline. The immunoprecipitates or cell pellets were boiled in reducing sample buffer and subjected to SDS-PAGE on 10% linear gels, which following drying were evaluated by autoradiography and densitometry as described (14). All incubations were performed in triplicates, and each experiment was repeated at least twice. Standard deviations were calculated according to the Student’s t-test from at least three different experiments.

Functional Competition Assay—The ability of the MAGE-1 peptide 161–169 and its photoreactive derivatives to bind to HLA-A1 was assessed in a recognition-based competition assay, as described previously (4, 13, 14). In brief, 125I-labeled C1R.A1 cells were incubated in the presence of a suboptimal concentration of the MAGE-3 peptide 168–176 with cloned HLA-A1-restricted MAGE-3-specific 20/38 CTLs (4). The concentration of the MAGE-1–161–169 peptide that resulted in 50% inhibition of the specific lysis was defined as 1, and the HLA-A1–specific CTL activities of the MAGE-1 peptide derivatives were expressed relative to this value.

Molecular Modeling—An average framework for the a1 and a2 domains of MHC class I molecules was constructed from structures available in the Protein Data Bank database (HLA-A2, HLA-B27, HLA-Aw68, and H-2Kb). Models for the a1 and a2 domains of other class I alleles were built from this framework using the ProMol knowledge-based modeling package (19). Briefly, a new carbon backbone was fitted onto the framework based on a primary sequence alignment optimized for three-dimensional similarity. Loop regions were reconstructed by structural homology searches through the Protein Data Bank, and missing side chains were rebuilt using a library of allowed rotamers. Similarly, an averaged framework for MHC-bound peptides was constructed from structures available from the Protein Data Bank, including the complexes of HLA-A2 with the peptides human immunodeficiency virus gp 120 195–207, hepatitis B nucleocapsid 18–27, influenza A matrix protein 58–66, human immunodeficiency virus reverse transcriptase 309–317, or human T-cell lymphotropic virus-1 reverse transcriptase 309–317, and H-2Kb with peptides vimentin intermediate filament protein 52–59 or Sendai virus nucleoprotein 324–332. Peptides of interest were fitted onto this framework using ProMod. The resulting crude models of MHC-peptide complexes were subjected to rigid body energy minimization, 200 steps of Powell minimization with constrained α-carbons, and 200 steps of Powell minimization without constraints, using the X-PLOR package with the PARAM 11 parameter set. In order to assess peptide conformations with potentially lower free energies, the following molecular dynamics simulations were used: the peptide–MHC complex was heated to 300 K in steps of 10 K, the peptide was allowed to move freely for 10–100 ps at this temperature, and the complex was cooled again to 0 K. As before, the X-PLOR package and the PARAM 11 parameter data set was used. The resulting models were subjected to additional refinement using the ICM software. Optimal conformations for side chains of both MHC and peptide were searched by a biased Monte Carlo procedure (20) at 2000 K, carried out simultaneously with local deformation of the peptide backbone and Brownian movements of the whole peptide molecule with an amplitude of 2 Å. For each model, 500,000 energy function simulations were calculated. The energetically lowest conformation for each model was considered as final. The final models were examined for consistency with known rules of peptide–MHC complex structure, such as hydrogen bond formation and electrostatic interactions of the terminal amino and carboxyl groups of the peptide, and the presence of the canonical peptide binding pockets on the floor of the peptide-binding site (21, 28).

RESULTS

Ability of Photoreactive MAGE-1 Peptide Derivatives to Bind to and to Photoaffinity Label HLA-A1—To identify an HLA-A1 photoprobe, derivatives of the MAGE-1 peptide 161–169 were prepared by single amino acid substitution with photoreactive Dap(ASA). As shown in Fig. 1A, all amino acids were substituted except the HLA-A1 anchor residues Asp-161 and Tyr-169. The ability of these conjugates to bind to HLA-A1 was assessed in a recognition-based competition assay. The concentration of the MAGE-1 peptide causing 50% inhibition of the lysis of 51Cr-labeled target cells sensitized with the MAGE-3 peptide 168–176, by an HLA-A1-restricted MAGE-3-specific CTL clone, was defined as 1. The competitor activities of the MAGE-1 peptide derivatives were expressed relative to this value. Only the derivative containing Dap(ASA) in P2 displayed a considerably (100-fold) reduced binding to HLA-A1 and thus was not further examined.

The remaining derivatives were tested for their ability to photoaffinity label HLA-A1 molecules on HLA-A1-transfected C1R cells (C1R.A1) (Fig. 1B). Following incubation of these cells with the radioiodinated peptide derivatives and UV irradiation, cell lysates were analyzed by SDS-PAGE. The derivatives containing Dap(ASA) in P1 or P7 efficiently labeled a material that migrated with an apparent M, of approximately...
Fig. 1. HLA-A1 binding of photoreactive derivatives of the MAGE-1 peptide. Photoreactive derivatives of the MAGE-1 peptide 161–169 (EADPTGHSY) were prepared by replacing each amino acid with Dap(IASA), except for the HLA-A1 contact residues Asp-163 and Tyr-169. The ability of these derivatives to bind to HLA-A1 was assessed in a recognition-based competition assay, and the HLA-A1 competitor activities are expressed relative to the MAGE-1 161–169 peptide (A). Alternatively, the radioiodinated peptide derivatives were incubated with HLA-A1-transfected C1R cells (C1R.A1), and following UV irradiation the lysates of the washed cells were analyzed by SDS-PAGE (10%, reducing conditions) (B).

45 kDa (lanes 1 and 5, respectively). The derivatives containing Dap(IASA) in P4 or P5 weakly labeled this component, whereas the remaining two derivatives failed to do so (lanes 2–4 and 6). This labeled material could be immunoprecipitated with the W6/32 mAb (data not shown). Since this mAb immunoprecipitates all HLA class I molecules (22) and HLA-A1 is the only HLA molecule significantly expressed by this C1R transfectant (18), this 45-kDa material is the HLA-A1 heavy chain. Indeed, untransfected C1R cells displayed no detectable HLA labeling (data not shown).

It is worth noting that the different MAGE-1 peptide derivatives also weakly labeled materials with apparent molecular mass of approximately 70, 96, and 150 kDa, respectively. It is conceivable that at least some of these are heat shock proteins, which have been reported to bind peptides (23). Since the different photoprobes labeled these species with different intensities relative to HLA-A1, it is likely that the underlying binding principles are different.

The MAGE-1 Peptide Derivative Dap(IASA)-ADPTGHLY Apparently Also Photoaffinity-labeled HLA-A29 and HLA-B44—The MAGE-1 peptide derivative Dap(IASA)-ADPTGHLY, which efficiently bound to and photoaffinity-labeled HLA-A1, was chosen to screen a panel of 14 lymphoblastoid B cell lines expressing over 44 different HLA class I molecules. These cells were subjected to the same labeling procedure as described for Fig. 1B. As shown in Fig. 2A, significant photoaffinity labeling of a 45-kDa material was observed only in the case of the EBV-transformed cell lines BM21 (lane 1), MOU (lane 4), LG2 (lane 11) and 807–02 (lane 15). Labeling of this 45-kDa material was not detectable on the other lines tested (lanes 2, 3, 5–10, and 12–14). The labeled materials were immunoprecipitable with W6/32 mAb and hence correspond to HLA class I heavy chains (data not shown).

While the HLA photoaffinity labeling on BM21 cells, which express HLA-A1, was expected, the labeling observed on MOU cells, which express HLA-A*2902, HLA-B*4403, and HLA-Cw*1601 (Fig. 2B), was unexpected. As suggested by the similarly efficient HLA labeling observed on 807–02 cells, which express HLA-A29 but not HLA-B44 or HLA-Cw*1601, this peptide derivative apparently also photoaffinity-labeled HLA-A29. HLA-Cw*1601 labeling could be ruled out, since COS-7 cells transfected with HLA-A1, but not with HLA-Cw*1601, displayed HLA labeling (lane 14 and data not shown). This is consistent with the finding that an HLA-Cw*1601-restricted MAGE-1 peptide (SAYGEPRKL) displayed no similarity with the HLA-A1-binding MAGE peptides (Ref. 24 and Fig. 3).

The weak labeling observed on LG2 cells (lane 11) suggested that Dap(IASA)-ADPTGHLY also photoaffinity-labeled HLA-B44. However, the possibility could not be ruled out that the labeled HLA molecule was another HLA-C allele that could not be typed by serology (Fig. 2A).

HLA-A29 and HLA-B44 Photoaffinity Labeling by Photoreactive MAGE-3 Peptide Derivatives—To obtain further information on the HLA photoaffinity labeling observed with Dap(IASA)-ADPTGHLY on LG2 and 807–02 cells, competition experiments were performed. Neither labeling was well inhibited by the parental MAGE-1 peptide 161–169, indicating that the Dap(IASA) modification of this peptide considerably increased its binding to the HLA molecules labeled on these cells. In addition, these experiments indicated that the MAGE-3 peptide 168–176 (EVDPIGHLY) was a better competitor than the MAGE-1 peptide 161–169 (data not shown). To find out whether the MAGE-1 161–169 and homologous peptides encoded by other MAGEs (Fig. 3) indeed bind to HLA-A29, we assessed the ability of all possible single Dap(IASA)-substituted MAGE-3 168–176 peptide derivatives to photoaffinity label HLA-A29 on C1R.A29 transfectants. Three derivatives efficiently photoaffinity-labeled HLA-A29, namely those containing Dap(IASA) in P1, P6, and P7, respectively. The labeling by EVDPIDap(IASA)HLY, but not by the other derivatives, was efficiently inhibited by the parental peptide (Fig. 4B and data not shown).

When tested on C1R.B*4403 cells, none of these derivatives efficiently labeled HLA-B*4403. Because the peptide-binding motif of HLA-B44 is glutamic acid in P2 and a C-terminal tyrosine or phenylalanine (25, 26) we repeated these experi-
ments with Dap(IASA) derivatives of the MAGE-3 decapptide 167–176 (MEVDPIGHLY). Significant HLA-B*4403 labeling was observed with the derivatives containing Dap(IASA) in P5 or P6 or best in P8. All labelings were inhibitable by the parental peptide (Fig. 4C and data not shown).

Ability of Homologous MAGE Peptides to Bind to HLA-A1, HLA-A29, and HLA-B44—The most frequently expressed MAGEs, MAGE-1, -2, -3, -4, -6, and -12, encode sequences homologous to the MAGE-1 sequence 161–169 (Fig. 3 and Ref. 1). To assess the ability of these MAGE nona- or decapptide to bind to HLA-A1, A29, and B44, the different HLA photoaffinity labeling systems were used. As shown in Fig. 4A HLA-A1 photoaffinity labeling on C1R.A1 cells by the MAGE-1 peptide derivative Dap(IASA)-ADPTGHSY was efficiently inhibited (around 98%) in the presence of a 100-fold molar excess of the homologous MAGE-1, -3, -4a, -4b, and -6 nonapeptides. In contrast, the MAGE-2 and -12 peptides, which lack an acidic residue in position 3, were only poor competitors. This was also true for the MAGE-1 decapptide. These results are in accordance with the known HLA-A1 binding motif, which is an acidic residue in position 3 and a C-terminal tyrosine (7–9). No significant inhibition was observed in the presence of the HLA-A2-restricted tyrosinase peptide 368–376. The failure of the tyrosinase peptide to affect the HLA-A1 photoaffinity labeling demonstrated that under these conditions free peptide does not detectably quench the photoaffinity labeling, as we have observed in other systems (13–15).

A considerably different pattern of inhibition was observed in the HLA-A29 system. As shown in Fig. 4B the MAGE-2, -3, -6, and -12 nonapeptides, at a 100-fold molar excess, inhibited the HLA-A29 photoaffinity labeling by the MAGE-3 nonapeptide derivative EVDP1-Dap(IASA)-HLY on C1R.A29 cells by 80–98%. Conversely, the MAGE-1, -4a, and -4b nonapeptides inhibited HLA-A29 photoaffinity labeling only weakly (34–60%), and the tyrosinase peptide again displayed no significant inhibition. As for HLA-A1, although less pronounced, the MAGE-1 decapptide bound less efficiently to HLA-A29 than the MAGE-1 nonapeptide. Similar differences were observed for other MAGE peptides (data not shown).

Finally, the HLA-B44 photoaffinity labeling on C1R.B*4403 cells by the MAGE-3 decapptide derivative MEVDPIG-Dap(IASA)-LY was used to assess the binding of the corresponding MAGE decapptides to HLA-B*4403. As shown in Fig. 4C the HLA-B*4403 labeling was inhibited in the presence of a 100-fold molar excess of the MAGE-2, -3, -6, and -12 peptides by 80–90%. The relatively inefficient inhibition of the HLA-B44 photoaffinity labeling by the parental MAGE-3 decapptide (86%) indicated that Dap(IASA) substitution in P8 artificially increased its binding to HLA-B44. The MAGE-1, -4a, and -4b peptides were less efficient competitors, causing only 52–63% inhibition. As shown for the MAGE-1 peptide in Fig. 4C, the MAGE decapptides were considerably more efficient competitors than the corresponding MAGE nonapeptides (63 versus 22% inhibition). This is in accordance with the peptide-binding motif for HLA-B44, which is glutamic acid in position 2 and tyrosine or phenylalanine at the C terminus (25, 26). Similar results were obtained when C1R.B*4402 cells were used instead of C1R.B*4403 cells (data not shown), indicating that the two main subtypes of HLA-B44 bind these MAGE peptides with similar efficiency. This is consistent with the observation

**Fig. 2.** Photoaffinity labeling of different cell lines with Dap(IASA)-ADPTGHSY. Fifteen different cell lines were subjected to photoaffinity labeling with Dap(125IASA)-ADPTGHSY and analyzed as described for Fig. 1B (A). The HLA class I molecule expression of the cell lines is summarized in panel B. The first nine were HLA homozygous EBV-transformed cell lines that have been described at the 10th International Histocompatibility Workshop (16) (workshop numbers are indicated as ws#), and their HLA-C expression has been determined by polymerase chain reaction (18). The heterozygous EBV-transformed cell lines were derived from HLA-typed individuals. In the case of HLA-C this serological typing was incomplete, as indicated by question marks. The remaining cell line was COS-7 cells transfected with HLA-Cw*1601.

**Fig. 3.** Partial amino acid sequences of the MAGE-1, MAGE-2, MAGE-3, MAGE-4a, MAGE-4b, MAGE-6, and MAGE-12-encoded antigens. The homologous sequences that bind to HLA-A1 and HLA-29 are shown in black boxes and gray cassettes, respectively, and the corresponding decapptides that bind to HLA-B44 are shown in black cassettes. The differences in the amino acid numbers among the different MAGE sequences originate from amino acid insertions or deletions in the first quarter of the sequences (1).
that these subtypes bind a very similar array of endogenous peptides (25).

MAGE-3 Peptide Binding by HLA-A1, HLA-A29, and HLA-B*4403 Involves Different Binding Principles—To obtain further information on the binding of MAGE-3 peptides by HLA-A1, A29, and B*4403, we examined several peptide variants. As shown in Fig. 5A the HLA-A1 photoaffinity labeling by Dap(IASA)-ADPTGHSY was inhibited in the presence of a 20-fold molar excess of the indicated MAGE peptide (A). Alternatively, analogous experiments were performed by using C1R.A29 cells and the MAGE-3 nonapeptide derivative EVDPI-Dap(IASA)-HLY (B) or C1R.B*4403 cells and the MAGE-3 decapetide derivative MEVDPIG-Dap(IASA)-LY (C). After UV irradiation the cells were detergent-lysed, the immunoprecipitated HLA molecules were analyzed by SDS-PAGE, and the resulting autoradiograms were evaluated by densitometry. All experiments were performed at least in triplicate. 100% of labeling refers to the labeling observed in the absence of a competitor peptide.

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C-terminal tyrosine with phenylalanine increased the MAGE-3 peptide’s binding to HLA-B44 nearly 2-fold, and replacement with leucine only slightly reduced the peptide binding. These findings are consistent with the reported HLA-B44 peptide-binding motif (glutamic acid in P2 and a C-terminal tyrosine or phenylalanine) and indicate that HLA-B44, unlike HLA-A1 and HLA-A29, can avidly bind also peptides with C-terminal residues other than tyrosine.

Computer Modeling of HLA-A1 and HLA-A29 and HLA-B*4403-MAGE-3 Peptide Complexes—To better understand in molecular terms the results obtained, computer models of complexes of HLA-A1, HLA-A29, and HLA-B44 with MAGE-3 peptides were built. According to our model of the HLA-A1-MAGE-3 peptide 168–176 complex the main anchoring of the peptide involves the binding of the Asp-170 side chain by the D pocket and of the Tyr-176 side chain by the F pocket of HLA-A1. As shown in Fig. 6A, HLA-A1 Arg-114 seems to play key role in both bindings. On one hand, it forms a salt bridge with MAGE-3 Asp-170, and on the other hand it forms a hydrogen bond with the phenol group of MAGE-3 Tyr-176. In addition, HLA Arg-114 forms a hydrogen bond with the carboxyl group of HLA-A1 Asp-116. It is conceivable that in a less favorable rotamer, this group can alternatively hydrogen-bond with the phenol group of the peptide tyrosine. Either kind of hydrogen bond formation stabilizes the peptide binding.

According to our modeling the MAGE-3 peptide 168–176 binding by HLA-A29 involves a similar binding of the peptide tyrosine by the F pocket (data not shown). This is consistent with the observation that HLA-A29 and HLA-A1 peptides both preferentially bind peptides containing a C-terminal peptide tyrosine (Fig. 5, A and B) and that both alleles have the same F pocket residues, except for the conservative substitution of residue 97 (Table I). According to our data, peptide binding by HLA-A29 is favored by a hydrophobic peptide residue in P2 (Fig. 5B). According to our model, the B pocket of HLA-A29 is formed in essence by the allele-specific HLA-A29 residues Ala-24, Met-45, Val-67, and Tyr-99 and is remarkably large and hydrophobic and thus seems to be adequate to effectively accommodate voluminous hydrophobic side chains (Fig. 6B).

In contrast, the B pocket of HLA-B*4403, according to our modeling, is very different (Fig. 6C). This pocket is more narrow and harbors the mostly polar HLA-B44 allele-specific residues Tyr-9, Thr-24, Lys-45, Ser-67, and Tyr-99 (Table I). Our data and data by others indicate that HLA-B44-binding peptides have a glutamic acid in P2 (Fig. 5C and Refs. 25 and 26). Our model suggests that the binding of this side chain by the HLA-B44 B pocket involves primarily the formation of a salt bridge with HLA-B44 Lys-45 and hydrogen bond formation with HLA-B44 Ser-67. Alternatively, in a different rotamer the carboxyl group of this glutamic acid can form hydrogen bonds with the phenol functions of HLA-B44 Tyr-9 and Tyr-99 (shown in green). In this case hydrogen bonding between HLA-B44...
Lys-45 and Glu-63 is expected to increase (data not shown). It is conceivable that in the bound state the peptide's glutamic acid flips back and forth between these two rotamers. This binding principle is different from one proposed by other investigators, according to which the glutamyl carboxyl group interacts simultaneously with HLA-B44 Lys-45, Ser-67, and Tyr-9 (26). According to both models optimal hydrogen bond formation can only be realized if the peptide residue in P2 is glutamic acid and not aspartic acid.

While the MAGE-3 peptide binding by all three HLA alleles under study involves the binding of the C-terminal peptide tyrosine side chain by the HLA F pocket, our modeling suggests that this interaction is different for HLA-B44 versus HLA-A1 and HLA-A29. As shown in Table I, the allele-specific F pocket residue 97 is Arg in HLA-B44 but Ile or Met in HLA-A1 and HLA-A29, respectively. According to our modeling this residue is in the vicinity of Asp-116, and both form hydrogen bonds with the phenol group of the C-terminal tyrosine (Fig. 6D). Additional modeling studies suggest that upon replacement of this tyrosine with phenylalanine a salt bridge is formed between Arg-97 and Asp-116, resulting in a reduction of the polarity at the bottom of the HLA-B44 F pocket (data not shown). In HLA-A1 and HLA-A29 such a neutralization is not possible (Fig. 6A and data not shown). This is consistent with the observation that peptide binding by HLA-A1 and HLA-A29 strongly prefers a C-terminal tyrosine, whereas HLA-B44 also efficiently binds peptides with C-terminal phenylalanine or even leucine (Fig. 5C and Refs. 25 and 26).

DISCUSSION

A main finding of the present study is that homologous MAGE peptides, most of which were previously known to bind to HLA-A1, can also bind to HLA-A29 and to HLA-B44 (Figs. 3 and 4). Overlapping peptide binding by different HLA class I alleles has been observed previously, allowing grouping of HLA alleles into supertypes. So far two HLA supertypes have been defined in this study (10–12). The cross-reactivity observed among the HLA molecules of these supertypes was essentially based on variations of a given HLA-peptide binding principle. In contrast, the overlapping peptide binding reported in this study clearly involves different binding principles.

Its only common feature was the binding of the C-terminal tyrosine of the MAGE peptides by the HLA F pocket. However, even this relatedness was limited in that the F pockets of HLA-A1 and HLA-A29 bind preferentially tyrosine, while the F pocket of HLA-B44 binds also phenylalanine or leucine (Fig. 5). However, the second main HLA-peptide contact was entirely different in the three systems. While for HLA-A29 and HLA-B44 this contact involved the binding of the peptide P2 residue side chain by the B pocket, as has been observed for many other HLA molecules, HLA-A1 instead binds the peptide P3 residue side chain by its D pocket (Figs. 4 and 5).

Peptide binding by HLA-A1 has been described previously in great detail (7–9) and our findings are consistent with these reports. All MAGE peptides under study that express the HLA-A1 peptide-binding motif (acidic residue in P3 and a C-terminal tyrosine) efficiently bound to HLA-A1, indicating that the contribution to the binding of the residues that are polymorphic in these sequences is not important (Figs. 3 and 4A). According to our model Arg-114 plays a key role in the peptide binding by HLA-A1 in that it is critically involved in the binding of the acidic peptide residue in P3 by the D pocket as well as the peptide tyrosine by the F pocket (Fig. 6A).

A different and more complex situation was observed for the MAGE-3 peptide binding by HLA-A29. Our competition experiments with MAGE-3 nonapeptide variants and modeling studies strongly suggest that this interaction involves the binding of the C-terminal tyrosine side chain by the F pocket and the peptide P2 residue side chain by a nonpolar B pocket. Regarding the latter interaction it is interesting to note that endogenous peptides eluted from the closely related HLA-A31, which has the same B pocket residues as HLA-A29 (Table I), predominantly contained Leu, Val, Phe, or Tyr in P2 (27). However, unlike HLA-A1, HLA-A29 bound the different MAGE peptides with considerably different efficiencies (Fig. 4B), suggesting that in this system secondary anchor interactions are more important. Together with HLA-A29 binding studies using MAGE-3 peptide variants (Fig. 5B) these data indicate that hydrophobic residues in P5 and P8 can strengthen peptide binding by HLA-A29. According to our modeling, these interactions are explained by hydrophobic interactions with equally nonpolar domains on the HLA-A29 surface, namely the D pocket and adjacent region on the α2 helix, as well as a region on the α1 helix flanking the F pocket.2

While further insights in HLA-A29 peptide binding have to await sequencing of endogenous peptides, we would like to add that in Caucasians the two main subtypes of HLA-A29 are HLA-A*2901 and HLA-A*2902, which differ only by one amino acid in position 19 (His in HLA-A*2901 and Asp in HLA-A*2902). Since this position is located in the last turn of the β-pleated sheet, thus remote from the HLA peptide-binding domain (21, 28), this substitution is unlikely to affect the peptide binding.

As seen from sequencing of endogenous peptides or binding studies with alanine-substituted peptides (Fig. 5D and Refs. 25 and 26) a hallmark of peptide binding by HLA-B44 is the requirement for a glutamic acid in P2. This also explains why the MAGE decapeptides bound to HLA-B44 more efficiently than the corresponding nonapeptides (shown for the MAGE-1 peptides in Fig. 4C) in that the MAGE nonapeptides lack one amino acid to undergo the stabilizing canonical interactions of the N terminus with the HLA molecule (21, 28). According to our model (Fig. 6C) and one published by DiBrino et al. (26) the

2I. F. Luescher, P. Romero, D. Kuznetsov, D. Rimoldi, P. Coulie, J.-C. Cerottini, and C. V. Jorgened, unpublished data.
HLA-B44 B pocket is ideally structured to avidly bind a glutamic acid side chain. Our modeling further proposes that in the F pocket of HLA-B44 Arg-97 and Asp-116 can either hydrogen bond with the phenol group of a C-terminal peptide tyrosine or, in the absence of this group, can form together a salt bridge (Fig. 6C). The resulting reduction of the polarity of the HLA-B44 F pocket provides an explanation for the observation that HLA-B44 also binds peptides with C-terminal Phe or even Leu (Fig. 5C, Refs. 25 and 26). It is interesting to note that several HLA-B alleles, such as HLA-B37, HLA-B40, HLA-B60, and HLA-B61, bind peptides containing Glu in P2 and a hydrophobic residue in PC (Table II). It is therefore conceivable that there may exist overlapping peptide binding among these HLA-B alleles (e.g. an HLA-B44 supertype).

While all the MAGE decapeptides under study have Glu in P2 and a C-terminal tyrosine (Fig. 3) and bind to HLA-B*4403, some variations in binding efficiency were observed (Fig. 4C). These differences are most likely explained by secondary anchor residues. For example sequencing of peptides eluted from HLA-B44 showed a preference for an aliphatic hydrophobic residue in P3 and often also in P6 (25, 26); this is consistent with the observation that the MAGE-1 and the two MAGE-4 decapeptides, which lack hydrophobic residues in these positions, bound less avidly to HLA-B44 (Figs. 3 and 4C). According to our modeling, residues in P3 and P6 undergo hydrophobic interactions with nonpolar domains on HLA-B44. It is noteworthy that the two major subtypes of HLA-B44 are HLA-B*4402 (nearly ½ in Caucasian populations) and HLA-B*4403 (about ½). HLA-B*4403 differs from HLA-B*4402 in having leucine, rather than aspartic acid, in position 156. Both subtypes are similar in their peptide binding (25). However, according to computer modeling, the conformation of HLA-B*4402 and HLA-B*4403 bound peptides can be significantly different, which may explain why HLA-B44-restricted CTLs generally recognize one or the other, but not both subtypes and why HLA-B44-allreactive T cell populations readily arise in donor/acceptor systems that differ in HLA-B44 subtypes (29).

Since the present study is the first to use photoaffinity labeling to analyze peptide binding by HLA class I molecules, the advantages and limitations of this approach are briefly discussed. As this technique utilizes purified iodinated photoprobe and 125I, it has a high specific radioactivity (around 2000 Ci/mM), and the ligand concentration in these experiments is low (nanomolar range). Therefore, this technique is mainly suitable for the study of avid HLA-peptide interactions (dissociation constants in the nanomolar range), which includes most, but not all, HLA class I-peptide interactions; otherwise, nonspecific labeling, based on random collision, will obscure the specific photoaffinity labeling. In the present study such problems were encountered only in the case of HLA-B44. These problems can be circumvented by using purified HLA molecules. This, however, voids the main advantage of this technique, which is photoaffinity on living cells. While these experiments are simple to perform, they require suitable radioactive photoreactive derivatives. While the synthesis of such compounds is usually easy (15), it is a priori not known in what position Dap(IASA) is best introduced in a given peptide, and thus several Dap(IASA) derivatives need first to be evaluated for efficiency and specificity of HLA photoaffinity labeling. One risk is that the photoreactive group in certain positions can significantly interact with the HLA molecule and increase the binding of the peptide derivative. In this situation the parental peptide is unable to efficiently inhibit the HLA photoaffinity labeling, as was observed in this study for the HLA-B44 photoaffinity labeling (Fig. 4C).

It is interesting to note that the MAGE sequences that display overlapping binding to HLA-A1, HLA-A29, and HLA-B44 constitute one of the most polymorphic regions in MAGE sequences (Fig. 3 and Ref. 1). This region is characterized by the presence of Glu-9, and usually Asp-7, residues before Tyr (Fig. 3). This constellation is essential for the observed cross-binding and is unique in the MAGE sequences. It is therefore conceivable that this region may be of special importance for the cellular immunity of MAGE-encoded tumor antigens.

From the MAGE peptides shown in Fig. 3 it was previously known that the MAGE-1, -3, -4a, -4b, and -6 nonapeptides bind to HLA-A1 (4–6) and that MAGE-1- and MAGE-3-specific CTLs can recognize the MAGE-1 161-169 and MAGE-3 168-176 peptides, respectively (4, 6). The present study shows that these, as well as the homologous peptides of MAGE-2 and 12, can also bind to HLA-B44 (Fig. 4). To find out whether this overlapping peptide binding is of immunological relevance, in vitro CTL induction experiments with MAGE-3 peptides were performed. Thus far we were able to induce with the MAGE-3 167-176 peptide HLA-B44-restricted CTLs that recognize MAGE-3 tumor cells. Since the genes that encode the peptides under study are the most frequently expressed MAGEs in tumor samples (1), the overlapping MAGE peptide binding described here suggests that immunotherapy of MAGE tumors may not be only applicable to HLA-A1 but also to HLA-A29 and HLA-B44 positive patients.

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