The generation of cytotoxic T cell epitopes and their generation for cancer immunotherapy

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CHAPTER 4

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Competition-Based Cellular Peptide Binding Assays for 13 Prevalent HLA Class I Alleles Using Fluorescein-Labeled Synthetic Peptides

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ABSTRACT: We report the development, validation, and application of competition-based peptide binding assays for 13 prevalent human leukocyte antigen (HLA) class I alleles. The assays are based on peptide binding to HLA molecules on living cells carrying the particular allele. Competition for binding between the test peptide of interest and a fluorescein-labeled HLA class I binding peptide is used as read out. The use of cell membrane–bound HLA class I molecules circumvents the need for laborious biochemical purification of these molecules in soluble form. Previously, we have applied this principle for HLA-A2 and HLA-A3. We now describe the assays for HLA-A1, HLA-A11, HLA-A24, HLA-A68, HLA-B7, HLA-B8, HLA-B14, HLA-B35, HLA-B60, HLA-B61, and HLA-B62. Together with HLA-A2 and HLA-A3, these alleles cover more than 95% of the Caucasian population. Several allele-specific parameters were determined for each assay. Using these assays, we identified novel HLA class I high-affinity binding peptides from HIVpol, p53, PRAME, and minor histocompatibility antigen HA-1. Thus these convenient and accurate peptide-binding assays will be useful for the identification of putative cytotoxic T lymphocyte epitopes presented on a diverse array of HLA class I molecules. *Human Immunology 64, 245–255 (2003).* © American Society for Histocompatibility and Immunogenetics, 2003. Published by Elsevier Science Inc.

KEYWORDS: peptide binding; HLA class I; MHC class I; fluorescent peptide; cellular peptide binding assay; competition

ABBREVIATIONS

HLA	human leukocyte antigen
aa	amino acid
β_2M	β_2-microglobulin
Fl-labeled	fluorescein-labeled
B-LCL	B-lymphoblastoid cell line
FCS	fetal calf serum
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
MF	mean fluorescence

INTRODUCTION

The identification of human leukocyte antigen (HLA)-restricted cytotoxic T lymphocyte (CTL) epitopes is crucial for our understanding of immunity in bacterial or viral infections, autoimmune diseases, and cancer as well as for the development of defined vaccines that induce CTL and the monitoring of such immunotherapies. The peptide-binding based prediction of CTL epitopes in protein sequences has led to the identification of CTL epitopes in viral proteins [1,2], bacterial proteins [3], and tumor antigens [4]. Often, these CTL epitopes are presented in HLA-A2, being the class I allele that predominates in the

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Caucasian population. However, an ongoing search for new CTL epitopes restricted by other prevalent HLA class I molecules is necessary for the development of immunotherapies covering all class I haplotypes and multi-epitope vaccines (Table 1).

The peptide-binding groove of HLA molecules contains highly polymorphic allele-specific pockets that accommodate side chains of the so-called anchor residues of the bound peptide [5, 6]. The peptide-binding groove of HLA class I molecules is closed at both sides [6] and thus HLA class I accommodates peptides with a length of 8–11 amino acids. Allele-specific peptide-binding motifs were defined by the analysis of naturally presented peptide pools eluted from class I molecules [7, 8]. Each HLA class I molecule displays a preference for certain aa at the major (primary) peptide anchor positions (relative position 2 and the C-terminus for most HLA class I molecules) that bind in the binding pockets. Amino acids at other positions in the peptide can significantly contribute to binding by their engagement in secondary pockets [9–16]. The knowledge of allele-specific peptide-binding motifs has led to the development of peptide-binding prediction algorithms by several groups [17–19]. Although these algorithms are extremely helpful to select potential HLA class I–binding peptides, experimental determination of the HLA class I–binding capacity is still considered necessary because of the partly undefined contributions to binding of each possible aa in every position of the peptide.

Peptide-HLA class I–binding assays employ either cell-bound class I molecules [20–28] or solubilized class I molecules [29–34]. Assays using cell-bound HLA class I molecules are either based on upregulation of class I molecules in processing defective cell lines [22, 23, 26] or on reconstitution of HLA class I molecules [24, 25, 27, 28]. Cell-free assays are quantitative and are based on competition for binding between a labeled reference peptide and a test peptide [32]. We previously applied the competition principle in easy-to-perform cell-bound HLA class I–binding assays for HLA-A2 and HLA-A3 [27]. In these assays, Epstein-Barr virus (EBV)-transformed B cell lines (B-LCLs) expressing the class I allele of interest are used, from which naturally bound class I peptides are eluted to obtain free class I molecules. Subsequently, B-LCLs are incubated with a mixture of a fluorescein (Fl)-labeled reference peptide, known to bind efficiently to the allele of interest, and titrated amounts of a competing test peptide. Cell-bound fluorescence is determined by flow cytometry and the inhibition of binding of the Fl-reference peptide is calculated as readout. We now report the adoption of this principle for an additional set of highly prevalent HLA class I alleles (HLA-A1, -A11, -A24, -A68, -B7, -B8, -B14, -B35, -B60, -B61, and -B62). Together with HLA-A2 and HLA-A3, these alleles cover more than 95% of the Caucasian populations. For each assay, the following allele-specific parameters were established: (1) a suitable reference peptide with known binding capacity for the allele; (2) the optimal position of the Fl-label in the reference peptide; (3) the required concentration of the labeled peptide; (4) the pH of the elution buffer used for acid stripping of class I molecules; (5) a B-LCL expressing the HLA class I molecule of interest; and (6) exclusion of binding of Fl-reference peptide to coexpressed class I molecules on the used B-LCL. The assays were used to identify several HLA class I–binding peptides derived from HIV-1pol, p53, PRAME, and minor histocompatibility antigen (mHag) HA-1. Finally, we analyzed the predictive power of a commonly used peptide-binding prediction algorithm for a set of HLA-A2–binding peptides to assess the need to actually assay the peptide-binding affinity after prediction of binding.

TABLE 1 Phenotype frequency distribution of HLA-I antigens for which assays were developed expressed as percentages among major populations[a]

HLA class I	Population			
	Black	Caucasoid	Asian	Amerindian
A1	9	26	7	11
A2	29	44	47	43
A3	13	22	6	8
A11	3	13	30	4
A24	6	20	42	52
A68	18	8	3	12
B7	15	17	7	5
B8	9	14	3	2
B14	7	6	1	3
B35	11	20	10	32
B60	1	6	17	5
B61	0	6	9	23
B62	2	8	16	21

[a] Phenotype frequencies for the HLA antigens have been deduced using the gene frequencies as given by Marsh *et al.* [38]).

MATERIALS AND METHODS

Cell Lines

The EBV-transformed B-LCL used for the binding assays were either obtained from the international histocompatibility workshop cell line repository or newly generated from peripheral blood mononuclear cells (PBMC) of healthy blood donors. All B-LCLs were cultured in complete culture medium consisting of IMDM (Biowhittaker, Verviers, Belgium) supplemented with 8% fetal calf serum (FCS) (Gibco BRL, Breda, The Netherlands), 100 IU/ml penicillin, and 2 mM L-glutamine.

TABLE 2 Allele-specific characteristics of HLA class I binding assays

HLA class allele[a]	Reference peptides used in the assays				Assay cell line		pH[e]
	Fl-labeled seq.[b]	[Fl-pep.]	Original seq.	Ref.[c]	Name	HLA class I type	
A1 (A*0l01)	YLEPAC (F1)AKY	150nM	YLEPAIAKY	32	CAA	A*0101, B*0801, CW*0701	3.1
A2 (A*0201)[d]	FLPSDC(F1)FPSV	150nM	FLPSDFFPSV	39	JY	A*0201, B*0702, CW*0702	3.2
A3 (A*0301)[d]	KVFPC(F1)ALINK	150nM	KVFPYALINK	32	EKR	A*0301, B*0702, Cw*0702	2.9
A11 (A*1101)	KVFPC(F1)ALINK	150nM	KVFPYALINK	32	BVR	A*1101, B*3501, Cw*0401	3.1
A24 (A*2402)	RYLKC(F1)QQLL	150nM	RYLKDQQLL	40	Vijf	A*2401, B*0702, Cw*0702	3.1
A68 (A*6801)	KTGGPIC(F1)KR	150nM	KTGGPIYKR	41	A68HI	A*6801, B*4402, Cw*0704	3.1
B7 (B*0702)	APAPAPC(F1)WPL	150nM	APAPAPSWPL	NP	JY	A*0201, B*0702, Cw*0702	3.1
B8 (B*0801)	FLRGRAC(F1)GL	50nM	FLRGRAYGL	42	Vavy	A*0101, B*0801, Cw*0701	3.1
B14 (B*1402)	DRYIHAC(F1)LL	150nM	DRYIHAVLL	43	CHE	A*2402, A*3301, B*1402, Cw*0201	4.0
B35 (B*3501)	NPDIVC(F1)YQY	150nM	NPDIVIYQY	44	BVR	A*1101, B*3501, Cw*0401	2.9
B60 (B*4001)	KESTC(F1)HLVL	125nM	KESTLHLVL	36	DKB	A*2402, B*4001, Cw*0304	3.1
B61 (B*4002)	GEFGGC(F1)GSV	50nM	GEFGGFGSV	36	Swei007	A*2902, B*4002, Cw*0202	3.1
B62 (B*1501)	YLGEFSC(F1)TY	150nM	YLGEFSITY	36	BSM	A*0201, B*1501, Cw*0304	2.9

[a] HLA class I allele of binding assay. Mostly, B-LCLs were used expressing the most common subtype of the allele (see HLA class I type).

[b] A nonanchor residue was substituted with a cysteine derivatized by a fluorescein group, denoted as C(F1).

[c] Most reference peptides were derived from the SYFPHEITI database (see ref. 19); here the original reference is cited. For HLA-B7, APAPAPSWPL (human p53 84-93) was found as high affinity binder in a molecular binding assay. NP = no published.

[d] Characteristics of HLA-A2 and HLA-A3 binding assays have been published before (ref. 27).

[e] Optimal pH of the elution buffer used for stripping naturally bound peptides.

Peptides

Peptides were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Syro II, Multi-Syntech, Witten, Germany) using Fmoc-chemistry. Peptides were analyzed by reversed-phase high performance liquid chromatography (HPLC) and mass spectrometry, dissolved in 50 μl dimethyl sulfoxide, diluted in 0.9% NaCl to a peptide concentration of 1 mM and stored at −20 °C until use. Fl-labeled reference peptides were synthesized as Cys-derivative. Labeling was performed with 5-(iodoacetamido)fluorescein (Fluka Chemie AG, Buchs, Switzerland) at pH 7.5 (Na-phosphate in water/acetonitrile 1:1 vol/vol). The labeled peptides were desalted over Sephadex G-10 and further purified by C18 RP-HPLC. Labeled peptides were analyzed by mass spectrometry.

Selection of Test Peptides for Binding Assays

Peptides derived from HIV-1pol, p53, PRAME, and mHag HA-1 that contain HLA class I peptide binding motifs were selected using either the BIMAS peptide-binding algorithm available via the Internet (http://bimas.cit.nih.gov/molbio/hla_bind/) [18] or an algorithm that was developed in our department [17].

Mild Acid Elution of HLA Class I–bound Peptides on B-LCL

Mild acid treatment of B-LCL to remove naturally HLA class I bound peptides was performed with minor modifications according to the principle first described by Sugawara *et al*. [35] and elaborated by our group [27]. B-LCLs were harvested and washed in phosphate buffered saline (PBS) and the pellet (2–15 × 10^6 cells) was put on ice for 5 minutes. The elution was performed by incubating the cells for exactly 90 seconds in ice-cold citric acid buffer (1:1 mixture of 0.263 M citric acid and 0.123 M Na_2HPO_4, adjusted to the pH listed in Table 2). Immediately thereafter, cells were buffered with ice-cold IMDM containing 2% FCS, washed once more in the same medium, and resuspended at a concentration of 4 × 10^5 cells/ml in IMDM medium containing 2% FCS and 2 μg/ml human β_2-microglobulin (β_2M) (Sigma, St. Louis, MO, USA).

HLA Class I Competition Binding Assays

Eight serial twofold dilutions of each competitor test peptide in PBS/BSA 0.5% were made (highest concentration 600 μM, sixfold assay concentration). In the assay, test peptides were tested from 100 μM to 0.8 μM. The Fl-labeled reference peptide was dissolved in PBS/BSA 0.5% at sixfold final assay concentration (see Table 2). In a well of a 96-well V-bottom plate, 25 μl of competitor peptide was mixed with 25 μl Fl-labeled reference peptide. Subsequently, the stripped B-LCLs were added at 4 × 10^4/well in 100 μl/well. After incubation for 24 h at 4 °C, cells were washed three times in PBS containing 1% BSA, fixed with 0.5% paraformaldehyde, and analyzed with FACScan flow cytometry (Becton Dickinson, San Jose, CA, USA) to measure the mean fluorescence (MF). The percentage inhibition of

Fl-labeled reference peptide binding was calculated using the following formula:

$$(1-(MF_{\text{reference + competitor peptide}} - MF_{\text{background}})/(MF_{\text{reference peptide}} - MF_{\text{background}})) \times 100\%$$

The binding affinity of competitor peptide is expressed as the concentration that inhibits 50% binding of the Fl-labeled reference peptide (IC_{50}). IC_{50} was calculated applying nonlinear regression analysis (with software CurveExpert 1.3, SPSS Science Software, Erkrath, Germany). An $IC_{50} \leq 5$ μM was considered high-affinity binding, 5 μM $< IC_{50} \leq 15$ μM was considered intermediate-affinity binding, 15 μM $< IC_{50} \leq 100$ μM was judged low-affinity binding, and $IC_{50} > 100$ μM was regarded as no binding. These IC_{50} cutoff values are based on our experience with HLA-A2 and HLA-A3 binding ligands and CTL epitopes [27].

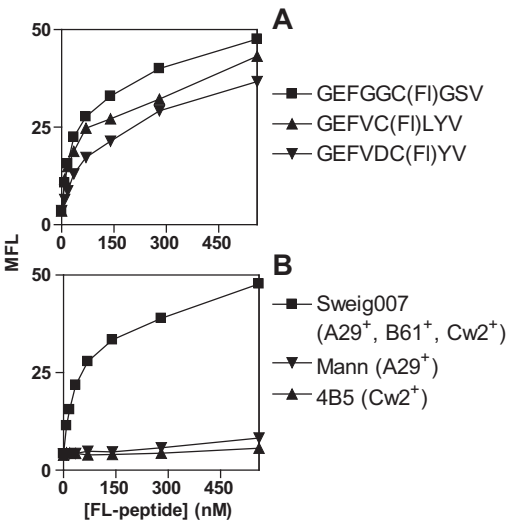

FIGURE 1 Determination of the optimal fluorescein (Fl)-labeled HLA-B61 binding reference peptide and exclusion of binding of the Fl-labeled peptide to alleles other than HLA-B61 that are expressed on B-LCL Swei007 (HLA I type: HLA-A29, -B61, and -Cw2). (A) Binding affinities of 3 Fl-labeled HLA-B61–binding reference peptides. The peptides were titrated at the indicated concentrations on B-LCL Swei007. After incubation for 24 hours at 4 °C fluorescence was measured with flow cytometry. (B) Exclusion of binding of the Fl-labeled reference peptide GEFGGC(Fl)GSV for HLA-B61 to coexpressed alleles on Swei007. The Fl-reference peptide was incubated for 24 hours at 4 °C with B-LCLs Swei007, Man (expressing HLA-A29), and 4B5 (expressing HLA-Cw2) and fluorescence was measured with flow cytometry at a FACScan. Results of one representative experiment of at least three performed are shown.

RESULTS

Selection of HLA Class I Alleles

The HLA-A2 and HLA-A3 alleles cover approximately 70% of the Caucasian population [27]. To enlarge the haplotype coverage, we chose to develop binding assays for 11 additional alleles (HLA-A1, -A11, -A24, -A68, -B7, -B8, -B14, -B35, -B60, -B61, and -B62) with high prevalence among most populations (Table 1). Together with HLA-A2 and HLA-A3, these alleles cover more than 95% of the Caucasian population, as calculated from a group of 1000 HLA-typed Dutch blood donors.

Selection of Optimal Fl-Labeled Reference Peptides

For each allele, one or two peptides to be used as Fl-labeled reference were derived from aa sequences shown to bind strongly to the particular allele. These peptides were originally identified as naturally presented class I ligand, CTL epitope, or consensus sequence, with the exception of the peptide for HLA-B7 (Table 2). For each peptide, several labeled variants were made by substituting at various positions a nonanchor residue for an Fl-conjugated cysteine. Fl-labeled reference peptides were titrated on B-LCL homozygously expressing the class I molecule of interest to identify for each allele the one that best retained its high binding capacity and to determine an optimal concentration of the Fl-labeled peptide to be used in the competition assay. As exemplified for HLA-B61, two peptides were selected: GEFGGFGSV (histone acetyltransferase 127-135 [36]), of which the phenylalanine at position 6 was substituted, rendering GEFGGC(Fl)GSV and GEFVDLYV (ribosomal protein S21 6-13 [36]), of which both GEFVC(Fl)LYV and GEFVDC(Fl)YV were tested (Figure 1A). Differences in binding capacity occurred depending on which original sequence was used and the particular residue that was substituted. The difference in binding capacity between the two variants of GEFVDLYV can be explained by altered contribution to overall binding affinity of the Fl-conjugated cysteine as compared with the original residue depending on the residue substituted and its position. The Fl-labeled reference peptide GEFGGC-(Fl)GSV, displaying highest binding capacity, was chosen as the labeled reference peptide for the assay (Figure 1A and Table 2). Optimal Fl-labeled reference peptides for the other alleles were likewise determined (Table 2). For each Fl-labeled peptide suboptimal saturating concentrations were used in the assay to optimally enable competition by the test peptides (Table 2). The maximal binding of Fl-labeled reference peptides at the chosen concentration after 24 h incubation at 4 °C resulted in a

MF of at least five times the background staining with PBS, as shown for HLA-B61 in Figures 1A and 1B.

Selection of HLA Class I Expressing Cell Lines

B-LCL homozygously expressing the allele of interest were used for the assays (Table 2). Control B-LCLs were tested to exclude binding of the Fl-labeled reference peptide to coexpressed class I molecules. As exemplified for the HLA-B61 binding assay, the Fl-labeled reference peptide GEFGGC(Fl)GSV did efficiently bind to B-LCL Sweig007 (HLA-A29, -B61, -Cw2), whereas binding to control B-LCL Mann and 4B5 expressing HLA-A29 and HLA-Cw2 respectively was absent (Figure 1B). This indicates that binding of the Fl-reference peptide to HLA-A29 and HLA-Cw2 can be excluded, and binding on Sweig007 was exclusively accomplished via binding to HLA-B61. B-LCL functioning optimally in assays for binding to HLA-A1, -A11, -A24, -A68, -B7, -B8, -B14, -B35, -B60, and -B62 were likewise found as listed in Table 2.

Determination of the Optimal Elution pH for Each Allele

We previously observed differences in the pH required for optimal elution of naturally bound peptides from HLA-A2 and HLA-A3 [27, 28]. Therefore, several pHs were tested for each new allele to find optimal conditions for removal of endogenous peptides, enabling efficient reconstitution of HLA class I–peptide complexes. For 8 of the 11 alleles for which novel assays were developed, elution at pH 3.1 produced the best results. However, for HLA-B14, -B35, and -B62, a different pH was chosen. For instance, elution of naturally presented peptides in HLA-B35 at pH 2.0 resulted in a considerable higher level of Fl-labeled reference peptide-binding than application of pH 2.4–4.0 (Figure 2). However, at pH lower than 2.8 cell viability decreased dramatically. We therefore chose pH 2.9 as an optimal compromise between these two phenomena. The optimal pH for every allele is listed in Table 2.

Optimization and Validation of the Competition Assays

A general improvement of the assay protocol was realized, compared with the published protocol [27], by adding FCS during incubation. Addition of 2% FCS improved binding of the Fl-labeled reference peptide (Figure 3) and increased cell viability from 30% to 90% after 24 hours (data not shown), which greatly enhanced cell recovery for FACS sample preparation. To validate each assay, the nonlabeled reference peptide or another positive control peptide, known from literature to be either a naturally presented ligand or CTL epitope, were tested in eight serial twofold dilutions

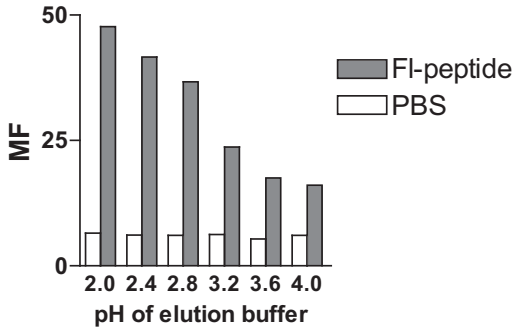

FIGURE 2 Determination of optimal pH of the elution buffer for HLA-B35. The elution buffer was adjusted to the various pHs and elution of naturally presented peptides from the surface of B-LCL BVR was performed at the indicated pHs as described in Material and Methods. Subsequently, B-LCL BVR was incubated with the HLA-B35 binding Fl-labeled reference peptide NPDIVC(Fl)YQY for 24 hours at 4 °C and fluorescence was measured with flow cytometry at a FACScan. Results of one representative experiment of at least three performed are shown.

(100–0.8 μM) for competition with the Fl-labeled peptide. The competition of binding of the HLA-B60 Fl-labeled reference peptide by the nonlabeled reference peptide KESTLHLVL is shown in Figure 4 as an example. Unmodified reference peptides and other positive control peptides were able to inhibit at least 50% of binding of the Fl-labeled reference peptide at concentrations lower than 5 μM ($IC_{50} < 5$ μM) (Table 3).

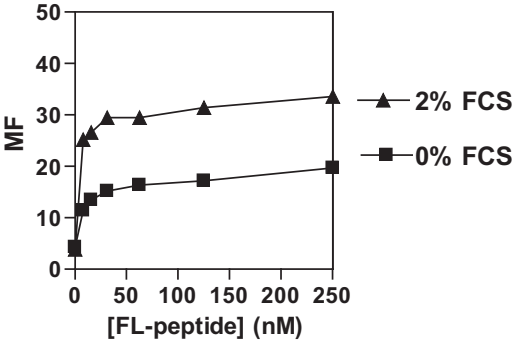

FIGURE 3 Influence of incubation without or with addition of 2% FCS on binding of the fluorescein (Fl)-labeled reference peptide. B-LCL JY expressing HLA-A*0201 was incubated with titrated amounts of the HLA-A2–binding Fl-labeled reference peptide FLPSDC(Fl)FPSV for 24 hours at 4 °C without or with addition of 2% FCS in the medium. Subsequently, fluorescence was measured with flow cytometry at a FACScan. Results of one representative experiment of at least three performed are shown.

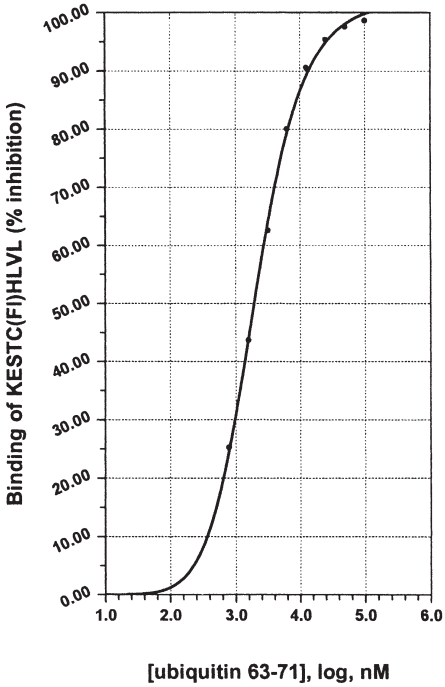

FIGURE 4 Competition of binding of the HLA-B60 fluorescein (Fl)-labeled reference peptide KESTC(Fl)HLVL by the nonlabeled original aa sequence KESTLHLVL to validate the HLA-B60 assay. The unlabeled peptide was titrated in 8 serial twofold dilutions (100 μM–0.8 μM) on B-LCL DKB (HLA-B60^{+}) together with the Fl-labeled peptide (125 nM) and was incubated for 24 hours at 4 °C. Fluorescence was measured with flow cytometry at a FACScan and the data were analyzed by regression analysis using software program CurveExpert 1.3 to determine the precise IC_{50} value expressed at a logarithmic scale. Results of one representative experiment of at least three performed are shown.

These results are in line with those obtained with high-affinity binding positive-control peptides in the published binding assays for HLA-A2 and HLA-A3 [27].

Identification of Novel HLA Class I Binding Peptides

The binding assays described herein were used for the identification of novel HLA class I–binding peptides derived from various protein sequences (HIV-1pol, p53, PRAME, mHag HA-1). For several alleles, candidate class I binding peptides were selected complying with the different HLA class I–binding motifs of interest, and their binding capacity was assessed. For these alleles, we successfully identified peptides binding with high or intermediate affinity (Table 4). Four peptides of HIV-1pol were found to bind with high affinity in HLA-A11 ($IC_{50} \leq 5$ μM), whereas one peptide displayed intermediate affinity (5 μM $< IC_{50} \leq 15$ μM). In HLA-A24, three peptides from HIV-1pol bound with high affinity, one peptide with intermediate affinity, and two with low affinity (15 μM $< IC_{50} \leq 100$ μM). Six peptides of p53 displayed high-affinity binding to HLA-B7. Seven of eight peptides derived from PRAME, predicted to bind in HLA-B35, displayed high binding affinity for this allele. Furthermore, we found four peptides of mHag HA-1 that bound with high affinity in HLA-B60 [37]. For the other alleles as well, several high-affinity binding peptides (derived from PRAME and BCR-ABL) were successfully identified by applying the present binding assays (manuscript in preparation). In summary, in all assays, peptides could be classified in the range from high-affinity binding to no observable binding affinity.

Correlation Between Peptide Binding Prediction and Peptide Binding Capacity

Although peptide binding prediction algorithms are extremely useful to select potential HLA class I–binding peptides, the currently prevailing view is that these predictions are not accurate enough to bypass binding measurements. We chose to analyze the binding prediction for HLA-A2 (-A*0201), because a refined binding motif is known for this extensively studied allele [10, 12, 18]. Previously, we identified 19 high- and 27 intermediate-affinity HLA-A2 binding peptides of tumor antigen PRAME (length 509 aa) of 65 nona- and 63 decamers selected [4] by using the BIMAS peptide-binding prediction algorithm [18]. Analysis of the data revealed that a relatively low prediction score did not necessarily exclude high-affinity binding. Examples of this group of peptides were decamers SLYSFPEPEA (PRAME 142-151) and FLKEGACDEL (PRAME 182-191) that ranked 35th and 46th in binding prediction for HLA-A2 (BIMAS algorithm), respectively (data not shown). Despite these low scores, SLYSFPEPEA bound second best (IC_{50} 1.9 μM), and FLKEGACDEL bound with high affinity as well (IC_{50}3 μM, ranking fifth for binding) [4]. Low prediction scores in these cases were caused by the lack of a canonical C-terminal anchor in SLYSFPEPEA and residues with a predicted deleterious effect on binding (E at P7 for SLYSFPEPEA and K at P3 for FLKEGACDEL). Conversely, a high prediction score for HLA-A2 did not necessarily correlate with high-affinity binding. Fifty percent of the predicted 16 best binding 9-mers and 18.7% of the 10-mers from the analogous group displayed only low or no binding affinity at all (Table 5). For instance, nonamer KMILKMVQL (PRAME 224-232) that ranked fifth in binding prediction for HLA-A2 actually failed to bind ($IC_{50} >$

TABLE 3 Positive control peptides used to validate binding assays

HLA class I allele	Positive control peptides[a]		
	Sequence	Source (ref.)	IC_{50} (μM)
HLA-A1	YLEPAIAKY	Consensus sequence (32)	
HLA-A2	FLPSDFFPSV	HBV cAg 18-27 (39)	0.5
	YIGEVLVSV	mHag HA-2 (45)	3.5
HLA-A3	KVFPCALINK	Consensus sequence (32)	0.7
	QVPLRPMTYK	HIV-1nef 73-82 (46)	0.2
HLA-A11	QVPLRPMTYK	HIV-1nef 73-82 (46)	2.0
	KQSSKALQR	BCR-ABL b3a2 (47)	5.7
HLA-A24	RYLKDQQLL	HIV-1env gp41 583-591 (40)	1.8
	AYIDNYKF	Consensus sequence (48)	0.6
HLA-A68	KTGGPIYKR	Influenza A NP 91-99 (41)	1.3
HLA-B7	APAPAPSWPL	Human p53 84-93 (not published)	0.5
	SPSVDKARAEL	Human SMCY 950-960 (mHag HY) (49)	0.7
HLA-B8	FLRGRAYGL	EBNA-3 339-347 (42)	0.2
	GFKQSSKAL	BCR-ABL b3a2 (47)	1.5
HLA-B14	ERYLKDQQL	HIV-1env gp41 584-592 (50)	7.5
HLA-B35	NPDIVIYQY	HIV-1 RT 330-338 (44)	1.2
HLA-B60	KESTLHLVL	Ubiquitin 63-71 (36)	1.9
HLA-B61	GEFGGFGSV	Histone acetyltransferase 127-135 (36)	0.2
	GEFVDLYV	40S ribosomal protein S21 6-13 (36)	0.3
HLA-B62	YLGEFSITY	40S ribosomal protein S15 114-122 (36)	0.6

[a] The unlabeled reference peptides were used as positive control peptide for all alleles except for HLA-A11 and -B13. For several alleles, additional positive control peptides were tested.

HLA = human leukocyte antigen.

100 μM) [4]. A possible explanation is that the strong deleterious effect on binding of glutamine at position 9 of the peptide [18] may also results from this aa in position 8 [12], but is not incorporated in the binding prediction score [18]. Taken together, we conclude that binding prediction for this particular set of peptides did not accurately correlate with binding affinities, confirming the need for actual peptide-binding assays.

DISCUSSION

Measurement of peptide HLA class I–binding affinity can be exploited for the identification of HLA class I–presented epitopes as is needed for, *e.g.*, vaccine development and insight in autoimmunity and graft-versus-host reactions. For these purposes especially HLA class I molecules with a prevalent distribution among different human populations are of interest (Table 1). The current report presents a concise summary of binding assays that were developed for 13 highly prevalent HLA class I molecules according to a competition-based strategy that uses a Fl-labeled class I–binding reference peptide and cell surface expressed HLA class I molecules. This type of binding assay has several advantages over molecular HLA class I–binding assays.

First, the assays are rapid and convenient, because there is no need for time-consuming production and purification of soluble HLA class I molecules. Furthermore, the readout is not dependent on either radioactive peptide labeling or conformation specific antibodies, of which the latter are not available for every allele, but instead on Fl-labeled reference peptides, the synthesis of which is straightforward. Finally, as equipment, a flow cytometer suffices.

We show that the concept of the assay can be adapted for basically every HLA class I allele of interest. Therefore, the present report can also be read as an instruction for the development of class I–binding assays that are still lacking. Several important allele-specific features need to be determined for each allele. Differences in binding capacity of the Fl-labeled reference peptides were observed depending on which residue was substituted for a Fl-labeled cysteine (Figure 1A). However, when a proper nonanchor residue was chosen for substitution (Figure 1A), the substitution did not appear detrimental for binding. Exceptionally, we met problems in finding a suitable Fl-labeled reference peptide. For example, we did not succeed thus far in obtaining a sufficiently binding Fl-labeled peptide for HLA-B44 (data not shown). We used B-LCL homozygously expressing HLA class I molecules of interest because B-LCLs are broadly available in the scientific community and can easily be generated from PBMC. The required exclusion of binding of the FL-labeled reference peptide to coexpressed alleles was accomplished with the use of properly selected nega-

TABLE 4 Identified HLA class I binding peptides

Allele	Sequence	Source	IC_{50} (μM)[a]
		HIV-1pol	
HLA-A11	AIKKKDSTK	221-229	4
	GIPHPAGLK	252-260	1
	QLDCTHLEGK	781-790	9
	AVFIHNFKP	898-946	2
	KIQNFRVYY	938-948	4
		HIV-1pol	
HLA-A24	FWEVQLGI	242-249	20
	RYQYNVLPQGW	298-309	1.3
	QYNVLPQGW	300-308	1
	PFLWMGYEL	381-389	1.2
	GYELHPDKW	386-394	20
	LWKGEGAVVI	957-966	6.5
		human p53	
HLA-B7	LPENNVLSPL	26-35	1.2
	SPALNKMFCQL	127-137	0.9
	RPILTIITL	249-257	0.2
	LPPGSTKRAL	299-308	0.2
	SPQPKKKPL	315-323	0.6
		PRAME	
HLA-B35	LPRELFPPL	48-56	0.7
	LPRRLFPPLF	48-57	1.6
	FPPLFMAAF	53-61	0.8
	RPRRWKLQV	113-121	>100
	IPVEVLVDLF	173-121	0.1
	LPTLAKFSPY	246-255	0.1
	CPHCGDRTFY	487–497	1.5
	EPILCPCFM	499-507	0.3
		mHag HA-1	
HLA-B60	KECVLHDDL	—	5.3
	KECVLRDDL	—	3.9
	KECVLHDDLL	—	1
	KECVLRDDLL	—	1.6

[a] Bindings affinity can be classified according to the following cutoffs. High affinity IC50 ≤ 5 μM; intermediate affinity 5 μM < IC50 ≤ 15 μM; low affinity; 15 μM < IC50 ≤ 100 μM; no binding IC50 > 100 μM.

HLA = human leukocyte antigen.

tive-control B-LCL (Figure 1B). As with in other competition-based assays, in our assays, the measured binding affinity of the test peptides is relative to the binding capacity of the Fl-labeled reference peptide. Therefore, we used well-defined HLA class I ligands or CTL epitopes as reference peptides (Table 2). As we have shown before for the HLA-A2 and HLA-A3 binding assays [27], the kinetics of peptide binding in our assays at 4 °C with an incubation time of 24 h followed the same pattern as those in assays applying soluble HLA molecules. Also, the ranking of peptides according to their IC_{50} was comparable to the ranking found in cell free-binding assays [27]. Validation of the newly developed assays with either the unlabeled reference peptide or other defined class I–binding peptides, showed IC_{50} values below or around 5 μM (Figure 3 and Table 3), which is in line with previously published results [27].

We were able to use the assays described for the identification of novel HLA class I–binding peptides as exemplified for HIVpol-derived peptides binding in HLA-A11 and HLA-A24, peptides of p53 with high affinity for HLA-B7, PRAME-derived peptides binding in HLA-B35, and peptides from mHag HA-1 with high affinity for HLA-B60 (Table 4). These peptides have been used for CTL inductions to identify new class I–presented epitopes [37].

An analysis of the motif-based peptide binding prediction in HLA-A2 revealed that rankings of the peptide-binding prediction and binding capacity (IC_{50}) did not accurately correlate (Table 5). This is caused by the incomplete knowledge of the contribution of each aa in every position of a peptide to HLA class I binding and,

TABLE 5 Accuracy of binding prediction in HLA-A*0201 of 128 peptides in PRAME

Binding prediction[a]		Binding affinity measured by HLA-A2 assay[c]			
Length	Ranking[b]	High	Intermediate	Low	No binding
9-mers	Ranked 1–16	6 (37.5%)	2 (12.5%)	6 (37.5%)	2 (12.4%)
	Ranked 17–32	1 (6.2%)	4 (25%)	10 (62.5%)	1 (6.3%)
	Ranked 33–48	1 (6.2%	4 (25%)	6 (37.5%)	5 (31.3%)
	Ranked 49-65	0 (0.0%)	0 (0.0%)	7 (41.0%)	10 (59.0%)
	Total (ranked 1–65)	8 (12.3%)	10 (15.4%)	29 (44.6%)	18 (27.7%)
10-mers	Ranked 1–16	6 (37.5%)	7 (43.7%)	3 (18.7%)	0 (0.0%)
	Ranked 17–32	1 (6.2%)	6 (37.5%)	7 (43.8%)	2 (12.5%)
	Ranked 33–48	4 (25%)	4 (25.0%)	5 (31.2%)	3 (18.7%)
	Ranked 491–63	0 (0.0%)	0 (0.0%)	6 (40.0%)	9 (60.0%)
	Total (ranked 1–63)	11 (17.5%)	17 (27.0%)	21 (33.3%)	14 (22.2%)

[a] Prediction by BIMAS algorithm, accessible via http://bimas.cit.nih.gov/molbio/hla_bind/ (ref.18).

[b] Ranking no. 1 is peptide with highest prediction score, which is predicted to bind best.

[c] Binding affinity classified according to the following cutoffs. High affinity: IC_{50} ≤ 6 μM; intermediate affinity; 6 μM < IC_{50} ≤ 15 μM; low affinity; 15 μM ≤ 100 μM; no binding IC_{50} > 100 μM.

HLA = human leukocyte antigen.

therefore, we consider actual peptide-binding assays compulsory for precise assessment of peptide-binding capacity to all HLA class I molecules. The currently presented peptide binding assays will be conveniently applicable for this purpose.

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