Development and Validation of a Fluorescence Polarization-Based Competitive Peptide-Binding Assay for HLA-A*0201—A New Tool for Epitope Discovery

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ABSTRACT: Various approaches are currently proposed to successfully develop therapies for the prevention and treatment of infectious diseases and cancer. One of the most promising approaches is the development of vaccines that elicit cytotoxic T lymphocyte (CTL) responses. Consequently, identification and exact definition of molecular parameters involved in peptide−MHC class-I interactions of putative CTL epitopes are of prime importance for the development of immunomodulating compounds. To better facilitate epitope discovery, we developed and validated a novel state-of-the-art biochemical HLA-A*0201 assay, which is comprised of technologically advanced cutting edge reagents. The technique is based on competition and uses a FITC-labeled reference peptide and highly purified soluble HLA-A*0201 molecules to quantitatively measure the binding capacity of nonlabeled peptide candidates. Detection by fluorescence polarization allows real-time measurement of binding ratios without separation steps. During standardization, the problem of assay parameter variation is discussed, showing the dramatic influence of HLA and reference peptide concentrations as well as the choice of the reference peptide itself on IC_{50} determinations. For validation, a panel of 15 well-defined HLA-A*0201 ligands from various sources covering a broad range of binding affinities was tested. Binding data were used to compare against pre-existing quantitative assay systems. The results obtained demonstrated significant correlation among assay procedures, suggesting that the application of fluorescence polarization in combination with recombinant sHLA molecules is highly advantageous for the accurate assessment of peptide binding. Furthermore, the assay also features high-throughput screening capacity, providing uniquely efficient means of identifying and evaluating immune target molecules.

The ability of the immune system to recognize virus-infected or cancerous cells has opened the door to the development of vaccines to treat or prevent various types of infectious diseases or cancers. An increased understanding of the mechanisms by which T cells recognize virus and tumor-specific antigens has stimulated much interest in the use of specific T cells as adoptive immunotherapy for infectious and malignant diseases. Particularly, $CD8^+$ T lymphocytes (CTLs)[1] play a critical role in controlling and/or eliminating these infected and neoplastic cells. They are implicated in immunity to not only pathogens such as viruses, which utilize the host cell's biosynthetic machinery to produce viral proteins (*1*−*11*), but also to parasitic and microbial invaders (*12*, *13*). CTL responses are likewise extended to include stimulation by aberrant proteins such as those associated with malignancies (*14*−*23*). In fact, class-I molecules have the potential of binding and presenting to CTLs any protein introduced into the endogenous processing pathway by either natural or artificial means (*24*−*29*). This knowledge serves as a motivating factor behind the development of peptide-based vaccines intended to elicit protective CTL responses to pathogens and other abnormalities, which otherwise remain cytoplasmically concealed from detection.

Peptide-based virus and tumor vaccination has been proven efficient in protecting against virus infection and tumor growth in many mouse experimental models and more recently in humans (*8*, *14*, *15*, *17*, *19*, *20*, *22*, *30*−*38*). This type of vaccination strategy offers many attractive features such as ease of manufacturing and characterization, as well as an excellent safety profile in past clinical studies by using defined epitopes, which are isolated from the context of the antigen of origin. The design of peptide-based vaccines has several potential advantages including not only a more potent response than that obtained by the use of whole antigens (*39*, *40*) but also allows the control over qualitative aspects of the immune response. Broad responses simultaneously targeting multiple dominant and subdominant epitopes can be induced (*36*, *41*). Accessing subdominant specificities to overcome T cell tolerance might be of particular value in

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[1] Abbreviations: CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; β2m, β-2 microglobulin; HLA, human leukocyte antigen; FP, fluorescence polarization; sHLA, soluble HLA; FITC, fluorescein isothiocyanate; DMF, dimethylformamide; BGG, bovine γ globulin; pFITC, FITC-labeled peptide; cps, counts per second; IC_{50}, inhibitory concentration; K_d, equilibrium dissociation constant; mP, millipolarization.

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the case of tumor antigens, thus facilitating the development of cancer vaccines even further (*14*, *42*).

CTLs recognize peptide fragments of cellular or viral proteins in the form of short peptides comprising 8−11 amino acids presented in association with major histocompatibility complex (MHC) class-I molecules on the surface of infected or neoplastic cells (*43*−*47*). These peptides are usually derived from intracellular protein pools and associated in the lumen of the endoplasmic reticulum with MHC class-I heavy-chain and β2-microglobulin (β2m) molecules, after which the MHC−peptide complex is transported to the cell surface. Considering the myriad of possible peptide sequences, coupled with the genetic variability of MHC molecules among individuals, the challenge of selecting the "right" target antigen or epitope presents a major obstacle in the development of such cell-mediated vaccines. Consequently, one of the major reasons to study peptide binding to class-I molecules is to be able to determine which peptides are likely to be antigenic, starting from the primary sequence of viral or cancer-related proteins. Although many factors influencing MHC class-I-restricted CTL responses to peptides have been elucidated, a major parameter determining cell-surface presentation of a given peptide is the affinity of the peptide for MHC class-I molecules (*48*, *49*). Recent reports show that peptides possessing good binding characteristics are a prerequisite for successful candidate CTL epitopes where remarkable association between the strength of binding and the immunogenicity of individual peptides in patients and HLA transgenic mice could be demonstrated. In this matter, several lines of evidence, both at the biological and functional level, emphasize the biologic relevance of peptide-binding assays in the identification and evaluation of potential peptide candidates (*48*, *50*−*56*).

Many different peptide−MHC-binding assays have been suggested over the years. The assays are performed using a number of different protocols with the common theme of assessing the relative abilities of synthetically defined peptides to associate with specific class-I heavy chains and β2m *in vitro* (*56*−*82*). However, most of them have been conducted under poorly controlled conditions, and only a few quantitative biochemical binding assays have been established (*56*, *57*, *66*, *79*, *80*, *82*, *83*). The most traditional biochemical binding assays utilize detergent-solubilized HLA class-I complex molecules in combination with iodinated peptides to measure peptide binding (*56*, *78*, *79*, *81*, *83*). In these conventional radioactive competition assays, radioactive iodine [^{125}I] is measured after chromatographic separation of bound from free peptide ligand (*56*, *77*, *79*). Unfortunately, this assay method involves multiple washes, liquid transfers, and incubation times that make it very labor-intensive and time-consuming. In addition to generating large volumes of radioactive waste, these steps cannot be completely automated. An additional disadvantage is that the complex is immobilized on an affinity matrix possibly interfering with the kinetics of the assay. A further limitation in most of these heterogeneous binding assay applications has been the lack of availability of high-quality class-I HLA proteins. In recent years, alternative competitive assay approaches have been developed using fluorescence labeled peptides in combination with cell-bound HLA class-I molecules from which naturally bound class-I peptides were eluted (*49*, *66*, *82*). In these cellular-based fluorescence procedures, binding events were detected by flow cytometry. However, these assays still consist of multiple steps and demand highly trained operators and expensive reagents, making them inherently slow and difficult to automate. Therefore, the improvement of reliable and quantitative assays will considerably aid in dissecting the quantitative aspects of class-I peptide interactions.

Table 1: Sequences of FITC-Labeled Peptide References Used[a]

name	sequence									
A*0201/P1	F	L	P	S	D	**K**	F	P	S	V
A*0201/P2	S	L	Y	N	**K**	V	A	T	L	
A*0201/P4	L	V	F	G	**K**	E	V	V	E	V
A*0201/P5	A	L	M	D	K	V	L	**K**	V	

[a] The amino acid lysine carrying the FITC-label is in bold font.

To overcome the difficulties of current assay procedures and further facilitate epitope discovery for MHC class-I molecules, we have developed and optimized a peptide competition assay based on the interaction between recombinant, soluble HLA molecule A*0201 and a fluorescence-labeled peptide reference using the technique of fluorescence polarization (FP). FP is unique among methods used to analyze molecular-binding events because it gives a direct, nearly instantaneous measurement of a ligand's bound/free ratio. The technique is based on the principle that small molecules rotate faster than large molecules. During binding, the small fluorescent peptide, which has free rotational mobility, is converted to a larger fluorescent peptide/HLA complex with restricted rotational mobility (*84*, *85*), resulting in an increase in polarization. Within the FP field, anisotropy values are sometimes preferred because it is easier to deconvolute anisotropy values into their component values than it is with polarization values. Polarization and anisotropy are both derived from the measured vertical and horizontal intensities. The values are mathematically related and easily interconverted [$A = 2P/(3 - P)$]. Both values represent a weighted average of the bound versus unbound states of the fluorescent molecule. However, it should be noted that, in the majority of applications, anisotropy does not give any more information than polarization and polarization values can be manipulated as if they were anisotropy values.

Our FP competition assay approach differs from other types of binding studies in one important regard: it requires no steps to separate the free from the bound tracer and is therefore fast, simple, and accurate. In this assay, an unlabeled peptide competes with a fluorescent-labeled peptide used as a tracer, whereas the usage of individual sHLA class-I molecules of defined specificity and purity offers the capacity for efficient standardization. This homogeneous assay is not only conceptually simple but also extremely sensitive and provides a viable option for easy automation, random sample accessibility, and simple adaption to existing instrumentation.

MATERIALS AND METHODS

Synthetic Peptides. FITC-labeled peptides were commercially synthesized by Synpep (Dublin, CA) or American Peptides (Sunnyvale, CA) using solid-phase strategies and purified with reverse-phase HPLC. The sequence of the reference peptides used for HLA-A*0201 are shown in Table 1, wherein a FITC-labeled lysine was introduced into the primary sequence of the native molecule as described earlier

(*86*). Unmodified unlabeled peptides were synthesized and purified by the Molecular Biology Resource Facility of the Warren Medical Research Institute at OUHSC (Oklahoma City, OK). Purity of all synthetic peptides was greater than 95%, and their composition was ascertained by mass spectrometric analysis. Peptides were stored dry in prealiquoted amounts of 0.2 and 0.4 μmol for labeled and unlabeled peptides, respectively. All lyophilized aliquots used in binding assays were originally dissolved in 100% DMSO or DMF at a concentration of 10 mM. Subsequent dilutions were done in 1× bovine γ globulin in phosphate-buffered saline (BGG/PBS; 0.5 mg/mL; 0.05%; Sigma, St. Louis, MO). Each pFITC preparation was standardized using a molar fluorescence intensity curve generated by a premeasured FITC standard (Pierce) reconstituted in 1× BGG (0.5 mg/mL). Aliquoted working dilutions for FITC-labeled peptides (200 μM) were kept at −20 °C and reused for a period of up to 4 months without a measurable decline in the signal.

Large-Scale Production and Purification of Recombinant sHLA Molecules. Production and purification of recombinant sHLA molecules was performed as described previously (*86*, *87*). Briefly, stable sHLA-transfected EBV-transformed B-lymphoblastoid cells (721.221) were expanded in RPMI-1640/20% FCS under 1.5 mg/mL G_{418} selection before seeding two hollow-fiber bioreactor units of the CP-2500 Cell Pharm System (Biovest International, Minneapolis, MN). After inoculation, a typical production run lasting between 4 and 6 weeks produced approximately 30−40 L of secreted sHLA product, which was collected as a crude harvest. Upon completion of a bioreactor run, sHLA complexes were affinity-purified from the harvest obtained using a Sepharose 4B-W6/32 matrix. Purified molecules were buffer-exchanged with PBS at pH 7.2 and concentrated using 10-kDa cutoff Macrosep centrifugal concentrators (Pall Filtron, Northborough, MA). The final product was filter-sterilized and stored at 4 °C until further use. The concentration of the purified molecules was determined using the Micro BCA protein assay kit (Pierce, Rockford, IL) using BGG as a standard. For subunit size confirmation and purity evaluation, SDS−PAGE was applied as quality control. The molecular weight of sHLA-A*0201 was determined to be 47.2 kDa, and the molecular weight of β2m was determined to be 11.7 kDa.

FP-Based Peptide Competition Assay. FP measurements were performed on an Analyst AD Assay Detection System (Molecular Devices; Sunnyvale, CA) using a continuous high-intensity, xenon-arc lamp as light source with filter settings suitable for FITC excitation (485 nm) and emission (530 nm). As a standard reading configuration, the excitation polarization filter was set in S (parallel) position (static), whereas the emission polarization filter was dynamically polarizing the light in either S or P (perpendicular) orientation. A fluorescein dichroic mirror (505 nm) was used to direct the polarized light into the assay well. Emitted polarized light was detected by the fluorescence photomultiplier tube with the SmartRead, sensitivity 2 setup option in counts per second (cps). Two intensity measurements were collected for each well, one when the dynamic polarizer was in S and one when the polarizer was in the P position.

For a standardized assay setup, each individual well of a black 96-well LJL HE PS microplate (Molecular Devices) was loaded with 5 μL of a 16× β2m solution (198.7 μg/mL; 16 936 nM) (Fitzgerald Industries International, Concord, MA), 10 μL of 4× competitor at various dilutions, and 5 μL of an 8× pFITC preparation (16 nM). For all preparations, 1× BGG/PBS was used as a buffer. To start peptide exchange, a 2× sHLA mix (100 μg/mL; 2117 nM) was activated by incubating at 53 °C for 15 min before adding 20 μL to the previously loaded wells reaching a final volume of 40 μL. After addition of all fluids, a final ratio [β2m]/[heavy chain] of 3:1 was achieved, where 2 parts of β2m are derived from the added β2m solution and 1 part from the sHLA complex. To remove potential air bubbles, the plate was spun down for 1 min at 2500 rpm. Note that the labeled and unlabeled ligand was presented simultaneously to the activated sHLA. The plate was incubated at room temperature and read periodically until no further increase in polarization was observed, indicating that equilibrium was reached (24−48 h). After each reading, the plate was covered with a lid and sealed with Parafilm to protect from light and to prevent evaporation of the constituents.

Specific control groups included (a) protein only, (b) tracer only, and (c) buffer only, which are used for background correction and calculation of the *G* factor as described earlier (*86*). The G factor {G factor = S/P(1 − (27/1000))/(1 + (27/1000))} is a scaling (correction) factor, taking relative polarization measurements and making them appear absolute. For this study, the theoretical value for free fluorescein (27 mP) was used for G-factor determination with a value of 1.00 ± 0.01. This value slightly dropped to 0.98 ± 0.02 for readings beyond 72 h, probably caused by minor evaporation of the reactant solution. Baseline polarization values determined for all pFITCs in their free state (average 30.3 ± 4.3) were not significantly different from the theoretical value used resulting in close to identical G-factor values. Once the G factor was determined, FP values given as mP (millipolarization) were calculated by the equation: polarization (mP) = 1000(S − GP)/(S + GP), where S and P are background-subtracted intensities of the fluorescence measured in the S and P directions.

For assay stability testing, plates containing binding experiments were measured at an extended time period of 3−7 days. No change in maximal polarization levels could be observed, indicating good stability of all constituents. As additional check on the system, the plates were also read in the fluorescence intensity mode to control fluorescent peptide concentrations according to the established standard curve for single peptides.

Competition Assay Data Analysis. Competition experiments were analyzed by plotting FP_{max} (maximal polarization) values as a function of the logarithms of competitor concentrations. The binding affinity of each competitor peptide was expressed as the concentration that inhibits 50% binding of the FITC-labeled reference peptide. Observed inhibitory concentrations (IC_{50}) were determined by nonlinear curve fitting to a dose−response model with a variable slope using the specific software Prism (Graph Pad Software, Inc., San Diego, CA). The appropriate values for FP_{max} were extracted from curve fittings of single-association reactions of FITC-labeled peptides to sHLA molecules in the presence of the competitor using a monoexponential association model as described earlier (*86*).

Table 2: Summary of Peptide Competition Data Obtained in Association with HLA-A*0201 Including Specific Information on the Peptides Origin and Position

sequence[a]	peptide origin[b]	position[c]	sequence ID[d]	peptide length (aa)	StD comp assay[e] (FP) IC_{50} (nM)	cell-free systems[f] (^{125}I) IC_{50} (nM)	references[g] (cell-free systems)	cellular systems[h] (fluorescence) IC_{50} (nM)	references[i] (cellular systems)
YLLPAIVHI	human; probable RNA-dependent helicase p72	146−154	Q92841	9	509.8	18	*131*		
YMDDVVLGA	hepatitis B virus (HBV); Pol	538−546	P03156	9	823.1	200	*40*, *95*, *97*		
FLPSDFFPSV	hepatitis B virus (HBV); core	18−27	P03146	10	870	0.57; 1.2; 2.5; 2.8; 3.0; 3.3[j]	*40*, *48*, *56*, *94*, *96*, *97*	400; 500[j]	*49*, *82*, *118*
YLVSFGVWI	hepatitis B virus (HBV); core	118−126	P03146	9	1742	1.9	*48*		
GILGFVFTL	influenza A virus; matrix protein M1	58−66	P21429	9	2247	6; 12.4[j]	*56*, *132*	4000	*100*
GLYSSTVPV	hepatitis B virus (HBV); Pol	61−69	P03156	9	2353	20; 33[j]	*48*, *99*	4500	*49*
KLGEFYNQMM	influenza B virus; NP	85−94	P13885	10	2820			5500	*49*
LLSSNLSWL	hepatitis B virus (HBV); Pol	407−415	P03156	9	3828	455	*99*	19 500	*49*
GLSRYVARL	hepatitis B virus (HBV); Pol	442−450	P03156	9	5113	71; 76; 79[j]	*40*, *97*, *99*		
SLYNTVATL	human immunodeficiency virus type 1 (HIV-1); Gag p17	77−85	P05888	9	6068	50	*133*	1500	*49*
ILKEPVHGV	human immunodeficiency virus type 1 (HIV-1); Pol	476−484	P03368	9	7082	192; 242[j]	*40*, *56*	8000	*49*, *100*
NLQSLTNLL	hepatitis B virus (HBV); Pol	400−408	P03156	9	9389	1000; 2000[j]	*48*, *99*	22 000	*49*
NLVPMVATV	cytomegalovirus (CMV); pp65	495−503	P06725	9	19 220			12 500	*100*
HLESLFTAV	hepatitis B virus (HBV); Pol	551−559	P03156	9	33 150	5000; 10 000[j]	*81*, *99*		
DLVHFASPL	hepatitis B virus (HBV); Pol	817−825	CAA46352	9	365 600	16 667	*81*		

[a] Nonlabeled parental peptide sequence synthesized for IC_{50} evaluation. [b] Protein source including species and category. [c] Peptide position within the original protein. [d] Swiss Prot reference link. [e] Data represent IC_{50} values obtained by fluorescence polarization using the standardized assay conditions described within the text. [f] Data represent IC_{50} values published for cell-free (HLA lysate) competition systems using ^{125}I as peptide tracer. [g] Peptide references for cell-free systems. [h] Data represent IC_{50} values published for cellular systems (cell-bound HLA) using a fluorescence-labeled peptide reference. [i] Peptide references for cellular systems. [j] Multiple values from different publications listed.

Epitope Screening. Experimental epitope screening at a threshold concentration of 80 μM was performed as described for the competition assay above with only minor modifications. Briefly, 10 μL of 4× competitor peptides (320 μM) (Table 2) were added to individual wells of a black 96-well microplate. Additional components such as 5 μL of an 16× β2m solution and 5 μL of an 8× pFITC preparation were added before starting peptide exchange with 20 μL of activated 2× sHLA solution, reaching a final volume of 40 μL. After equilibrium was reached, FP values were collected on the Analyst AD (Molecular Devices) and transformed into percent inhibition values by choosing the reaction without the inhibitor as 0% inhibition.

RESULTS

Competition binding assay methodologies have become exceedingly popular for assessing the ability of synthetically defined peptide epitopes to associate with specific HLA class-I complexes. This has been accomplished by comparing the relative affinities of multiple peptide ligands for the same HLA receptor. Experimentally, IC_{50} values, representative of the affinity of the ligand-recognition site, have been determined by incubating HLA molecules from various sources with a labeled peptide in the presence of different concentrations of the competitor. However, results presented from various assay systems have shown a high variability rate, and thus, difficulties have been encountered in obtaining reproducible data. To further enhance the capabilities of such assays and better facilitate epitope discovery for MHC class-I molecules, we have developed a novel peptide competition assay based on the interaction between recombinant, sHLA molecules A*0201 and a fluorescence-labeled peptide reference using the technique of fluorescence polarization. To demonstrate feasibility but also increase the sensitivity and reproducibility of the assay, we initially conducted a detailed analysis of all parameters critical for the standardization of the assay. This analysis was also intended to elucidate critical factors for interassay data comparisons.

Influence of Different Reference Peptides on IC_{50} Values. A primary requirement for the development of a more advanced competition assay is the careful selection of a reference peptide. Recently, we demonstrated the binding characteristics of several pFITC ligands to sHLA-A*0201 through kinetics experiments, which seem to be well-suited to act as a reference peptide (*86*). The labeled peptide candidates were derived from peptides originally identified as naturally presented class-I ligands, CTL epitopes, or constructed from the A*0201 consensus sequence (*86*). All designs were made by substituting at various positions at nonanchor residues for a FITC-conjugated lysine (Table 1).

To visualize the effect of pFITC molecules with different equilibrium dissociation constants (K_d) on IC_{50} determinations, we performed FP-based competition assays using a constant concentration of activated sHLA (40 μg/mL; 847 nM), excess β2m (1694 nM), and labeled peptide (10 nM) in the presence of different concentrations of the unlabeled ligand KLGEFYNQMM as a test competitor (Figure 1). The selected A2-related competitor peptide was derived from the influenza B virus (NP; 85−94) chosen because of its successful use in several HLA studies (*49*, *73*, *88*, *89*). For each of the assays shown in Figure 1, bound fluorescent peptide was displaced from the sHLA molecule. The top of each curve represents a plateau at a value equal to binding in the absence of the competing unlabeled peptide that was set as 100% binding. Because a log axis cannot accommodate a concentration of zero [log(0) is undefined], a value for a very low competitor concentration was entered (−2). The bottom of the curve is a plateau equal to the values for free labeled peptide, which indicates almost complete competition at higher concentrations of the competitor (set as 0%

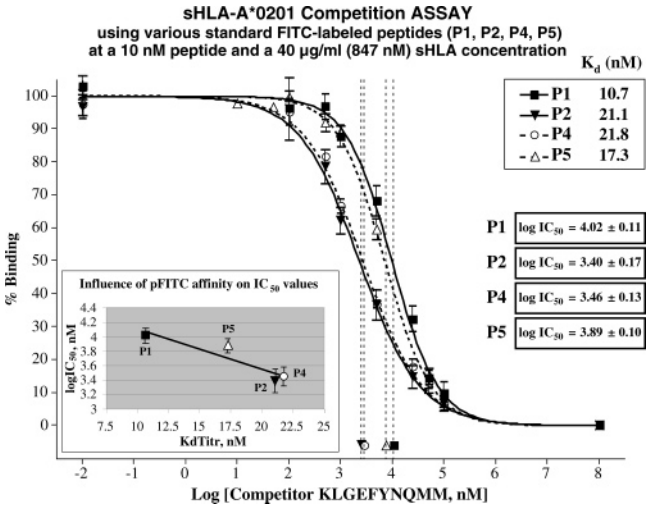

FIGURE 1: Soluble HLA-A*0201 competition assays testing the influence of various pFITC reference peptides on the IC_{50} value for the unlabeled competitor KLGEFYNQMM. Serial dilutions of the competitor were prepared in 96-well plates and incubated with a constant concentration of activated sHLA-A*0201, excess β2m, and FlTC-labeled reference peptides P1, P2, P4, and P5. After equilibrium was reached at room temperature, the FP of each well was read on an Analyst AD using a filter set appropriate for fluorescein. The IC_{50} for the competitor was calculated from nonlinear regression analysis using the software program Prism. The *x* axis plots the logarithm of the concentration of the test peptide, where the *y* axis plots the response expressed in percent binding of the pFITC to sHLA. To better compare data, maximum and minimum responses were normalized expressing FP_{max} as 100% and FP_{min} (27 mP) as 0% binding. The IC_{50} concentrations of the unlabeled competitor that produces pFITC binding halfway between the upper and lower plateaus are indicated. A linear relationship (inset) is shown plotting IC_{50} values as a function of the various K_d values of the pFITC reference peptides.

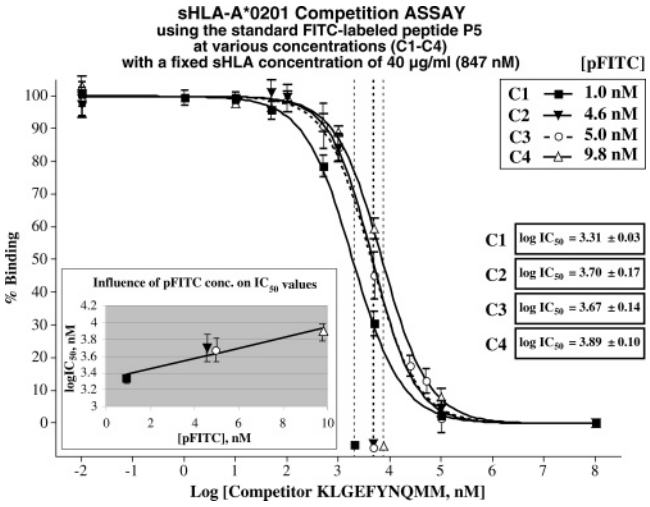

FIGURE 2: Soluble HLA-A*0201 competition assays testing the influence of different pFITC concentrations on the IC_{50} value of the unlabeled competitor peptide KLGEFYNQMM. Serial dilutions of the competitor were incubated together with a constant concentration of activated sHLA-A*0201, excess β2m, and the FlTC-labeled reference peptide P5 at concentrations of 1.0, 5.0, and 10 nM. After equilibrium was reached at room temperature, polarization values were read and IC_{50} values for the competitor were calculated. A linear relationship (inset) was found by plotting IC_{50} values as a function of the pFITC concentration.

binding). Because of the usage of the G factor, which corrects for the contribution of the measurement pathway to the observed total polarization, the value for nonspecific binding (0%) is equal to the assumed theoretical value of 27 mP. High ratios between the maximum and minimum signals were observed for all competition experiments, with maximal FP values from 183.3 to 224.9 mP depending on the pFITC used (data not shown).

The concentration of the unlabeled competitor that produced 50% inhibition of fluorescent-labeled peptide binding (halfway between the upper and lower plateaus of the obtained curve) and defined as the inhibitory concentration (IC_{50}) was calculated by nonlinear regression analysis using the software program Prism. The obtained IC_{50} values for the labeled pFITC ligands ranged from 2480 to 10 500 nM, demonstrating the specific nature of the interaction. No competition was observed testing a nonrelated peptide over the same concentration range used (data not shown). When the IC_{50} values were plotted as a function of the various K_d values of the pFITC tracers, a linear relationship was found (inset of Figure 1), confirming that the competitive displacement of a reference peptide from its binding site depends on the affinity of the labeled pFITC ligand (*90*, *91*). Conclusively, it takes more unlabeled competitor peptide (high IC_{50}) to compete for a tightly bound tracer peptide (low K_d) than for a loosely bound tracer peptide (high K_d), which makes it mandatory to assign a single peptide to be used as the reference peptide in a standardized assay environment.

Influence of pFITC Concentrations on IC_{50} Values. To fully assess the influence of the pFITC concentration on IC_{50} values in FP-based competition assays, we tested our model competitor KLGEFYNQMM at several nanomolar concentrations of pFITC P5 utilizing a constant concentration of 40 μg/mL (847 nM) activated sHLA and excess β2m (1694 nM) (Figure 2). Final analysis showed declining IC_{50} values with reducing pFITC concentrations confirming a linear relationship between the fluorescent-labeled tracer peptide concentration and IC_{50} determinations (*90*−*92*) (inset of Figure 2). As seen, choosing a higher concentration of tracer will take a larger concentration of unlabeled peptide to compete for half the binding sites. Therefore, to achieve higher accuracy and consistency during the assay performance, working concentrations of pFITC reference peptides have to be standardized and constantly controlled.

Influence of sHLA Concentrations on IC_{50} Values. The effect of the sHLA concentration on binding competition curves for the unlabeled peptide KLGEFYNQMM is shown in Figure 3A. It is evident that increasing the concentration of sHLA caused a significant shift of the competition curve to the right, considerably increasing the concentration of unlabeled competitor required to inhibit 50% of the binding of the FITC-labeled reference peptide P5. Regression analysis showed that the IC_{50} values obtained from the competition binding curves vary as a linear function of the receptor concentration (inset of Figure 3). Doubling the sHLA concentration caused nearly a 2.5 times increase in IC_{50} values for peptide KLGEFYNQMM. Results were further elaborated by testing the peptide FLPSDFFPSV under equal conditions (Figure 3B). This competitor peptide was derived from an epitope located between residues 18−27 of the hepatitis B nucleocapsid core protein and known to bind A*0201 with high affinity (*34*, *56*, *81*, *93*). Indeed, results differ in that the regression lines do not concur to each other and the *x* variable for peptide KLGEFYNQMM is twice of

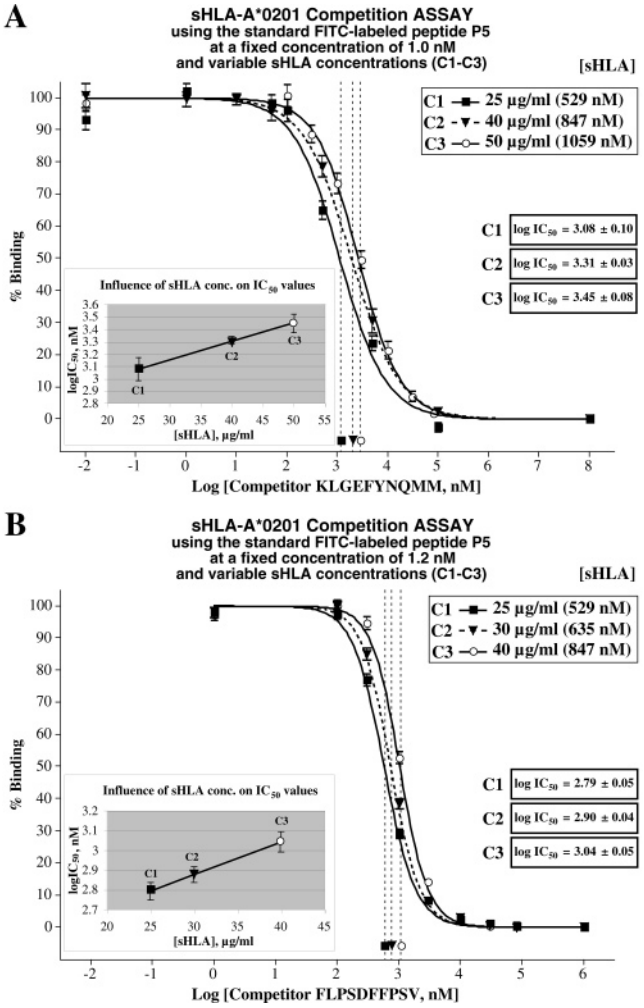

FIGURE 3: Effect of the sHLA concentration on competition binding data. Competition binding curves were obtained when the FP readout adjusted to percent binding was plotted as a function of the competitor concentration. Experiments were performed using variable sHLA-A*0201 concentrations with excess β2m, 1 nM fluorescent peptide P5, and increasing amounts of either (A) competitor peptide KLGEFYNQMM or (B) competitor peptide FLPSDFFPSV. Data were collected, and IC_{50} values were calculated by the nonlinear curve-fitting program in Prism. Insets show the linear relationship between sHLA and IC_{50} determinations.

that for peptide FLPSDFFPSV, indicating that the FLPSDFFPSV affinity is 2-fold higher than KLGEFYNQMM for sHLA-A*0201. A direct comparison of IC_{50} values at identical sHLA concentrations confirmed these findings. Our results clearly demonstrate the effect of the HLA component on the assay outcome and show the necessity of utilizing high-quality HLA molecules, which have to be applied under standardized conditions to guarantee reproducibility.

DMSO/DMF Tolerance and Assay Stability. HLA-A2 peptides are quit-biased toward hydrophobicity, given the hydrophobic peptide-binding pocket of the HLA-A2 molecule and the preference for hydrophobic residues throughout. This bias inevitably leads to differences in solubility and, therefore, bioavailability in the assay. Therefore, dissolving HLA peptide candidates is a critical first step in a successful screening application because the wrong choice of solvent can lead to inaccurate or complete lack of performance. One of the most powerful solvents currently used, which is able to minimize these effects, is DMSO, widely accepted in many end-use applications for peptides. However, under certain

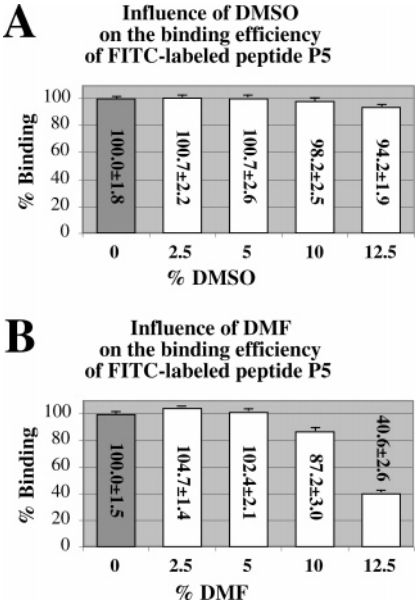

FIGURE 4: Effects of (A) DMSO and (B) DMF on the signal output for the sHLA-A*0201 and pFITC P5 interaction. Detergent (0−12.5%) was incubated with FP assay solution containing 1 nM fluorescein-labeled peptide P5, 50 μg/mL (1059 nM) sHLA, and excess β2m (2117 nM). The FP signal was read after reactions reached equilibrium. As seen, the assay can withstand detergent concentrations of up to 5% without any perceptible effect on pFITC binding.

circumstances, DMSO can be a weak oxidizing agent, and its use may result in gradual oxidation of C-containing peptides to dimers or polymers through disulfide bridge formation during long-term storage. Peptides containing M or W in their primary sequences may also be affected. For these particular cases, DMF replaced DMSO helping to avoid such oxidation effects. Accordingly, all peptides used for IC_{50} determinations were diluted following these guidelines.

Because screening platforms must be capable of withstanding DMSO/DMF concentrations of 1−5%, sensitivity of the FP signal to DMSO and DMF was quantified (Figure 4). The FP assay for the interaction of pFITC peptide P5 was chosen as a model system to test the effect of these solvents using concentrations between 0 and 12.5%. Results showed that concentrations up to 5% of DMSO or DMF had no significant effect on maximum polarization and are well-tolerated by comparing maximum signals with control reactions free of solvent. At higher concentrations, the FP signal was nearly insensitive to DMSO up to 12.5%, whereas DMF detectably influenced the binding interaction of sHLA with the fluorescein-labeled peptide, resulting in a decrease in the FP signal to 40% binding. Nonetheless, both solvents tested for our approach were found to be highly compatible with the assay. For overall quality assurance, dilution schemes for competitors were chosen so that the highest concentration of a titration did not exceed a final assay concentration of 4% DMSO/DMF.

During assay development, it was also of interest to determine complex stability under applied assay conditions, a critical aspect in approaching high-throughput applications. Therefore, a variety of competition reactions were chosen to test for the stability of the maximal FP signal over time (data not graphed). The plates were incubated at room temperature, and FP was measured over a period of 7 days.

Typically, the assay reactions reached equilibrium within the first 24−48 h, and no further change in polarization signal or calculated IC_{50} value was detected. For plates that were not carefully sealed with Parafilm, a slight increase of signal could be observed because of evaporation of the reaction solution. However, this evaporation artifact had no visual effect on the IC_{50} value itself. Ultimately, the results showed that the assay is extremely robust, because IC_{50} values remained unchanged during the investigation period of 7 days.

Determination of IC_{50} Values on Established Allele-Specific Test Peptides. At this point, a standardized procedure was applied and optimized according to the results obtained from our titration experiments, which clearly demonstrated that IC_{50} values depend on (1) the affinity, (2) the concentration of the tracer peptide, (3) the amount of binding sites available (sHLA concentration), as well as (4) the affinity of the competitor itself. To achieve maximal performance in sensitivity and accuracy, the FITC-labeled reference peptide P5 ALMDKVL-K(FITC)-V was selected, which showed the largest dynamic range between the bound and the free fluorescent-labeled peptide, thus allowing the sensitive detection of small decreases in polarization when adding a competitor peptide. For the procedure, a concentration of 1 nM of FITC-labeled peptide was adopted, which lies within the dynamic range of the FP detector and showed minimal signal fluctuations. Finally, to obtain consistent affinity values, a fixed amount of 50 μg/mL (1059 nM) sHLA-A*0201 was selected for all validation experiments. The addition of a 2-fold excess of β2m (2117 nM) over sHLA resulting in a β2m/heavy-chain ratio of 3:1 was established recently as the most optimal combination for maximum signal output (*86*) and was automatically included in all experiments throughout this study. Competitor peptides were typically tested at concentrations ranging from 100 nM to 80 μM. Data were visualized by plotting the logarithm of the concentration of the competitor on the *x* axis, where the *y* axis plotted the response, which were changes in fluorescence polarization (mP) monitoring the binding of the reference peptide to sHLA-A*0201. All IC_{50} values were calculated applying nonlinear regression analysis by fitting the inhibition data to a dose−response model as described earlier in the text.

Because FP has no actual zero value because the minimum polarization depends on the fluorescent ligand used (27 mP for fluorescein), signal-to-noise ratios were calculated by interpreting noise as the variability in measured FP and interpreting signal as the maximum change in FP. Typically, a ∼8 mP standard deviation in replicate assay wells compared to a maximal signal of 220−230 mP was achieved. This low noise compared to the maximal signal allows for sensitive detection of the effects of competitors and thus accurate determination of potencies. Experiments performed under suboptimal conditions, as seen in Figures 1−3, showed somewhat higher deviation values of ∼12 mP. They are expressed in percentages of maximal binding.

For our proposed validation approach, we selected a panel of 15 peptides to represent binders that reflect several orders of magnitude of A*0201 binding affinity (Table 2). The peptides were derived from various sources, including HBV nucleocapsid- and polymerase-derived peptides, two variants of a CTL epitope from HIV-1, the influenza matrix protein

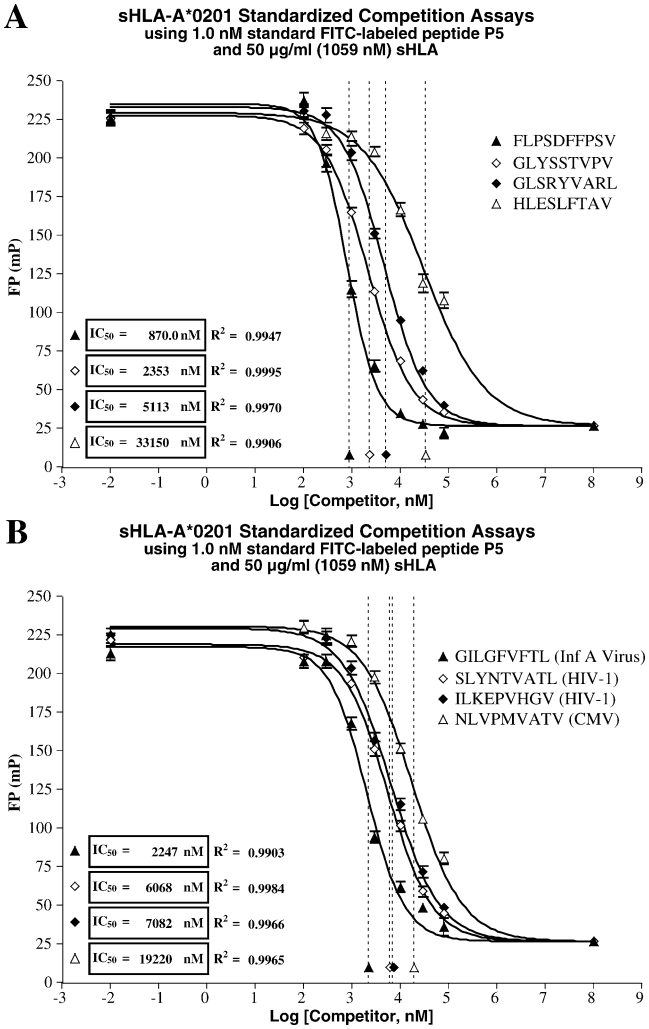

FIGURE 5: Standardized competition assays for sHLA-A*0201. Peptides from HBV (A) and multiple other viral sources (B) were tested with our standardized peptide competition assay for sHLA-A*0201. The unlabeled peptides were titrated in 8 serial dilutions (100 nM−80 μM) in competition with the FITC-labeled peptide P5. Fluorescence polarization expressed in mP was measured with the Analyst AD. IC_{50} values for the peptides were determined by fitting the data to a dose−response model using the software Prism. Because a log axis cannot accommodate a concentration of zero [log(0) is undefined], a value for a very low competitor concentration was entered (−2). The value for nonspecific binding was set to be equal to the assumed theoretical value of 27 mP defining the bottom of the curve. Results of one representative experiment of at least three performed are shown. R^2 values indicate the goodness of fit.

Flu-M1 peptide, as well as several other peptides of which the relative binding affinity for HLA-A*0201 was reported previously. Because each competition assay system is generally defined by different internal parameters, such control peptides had to be carefully selected. Only candidates previously evaluated through direct cell-free I^{125}-radioligand assay systems (*40*, *48*, *51*, *56*, *94*−*99*) or through cellular-based fluorescence assay procedures (*49*, *82*, *100*) were accepted, with the additional constraint that IC_{50} determinations for each system were performed under similar assay conditions. For some peptides, more than one IC_{50} value was found. In the FP evaluation process, each test competitor generated its own binding isotherm from which IC_{50} values were determined. Parts A and B of Figure 5 present a selection of multiple reaction curves obtained from the

Table 3: Definition of IC_{50} Categories for Different Assay Systems

assay systems	category						references
	high affinity	medium affinity	low affinity	very low affinity	no affinity		
StD FP comp assay	< 5000	5000 50 000	50 000 350 000	350 000 1 000 000	1 000 000 >	IC_{50} (nM)	
	< 3.7	3.7 4.7	4.7 5.5	5.5 6.0	6.0 >	log(IC_{50}; nM)	
cell-free I^{125} radioassays[a]	< 50	50 500	500 50 000		50 000 >	IC_{50} (nM)	*34*, *48*, *81*, *99*
	< 1.7	1.7 2.7	2.7 4.7		4.7 >	log(IC_{50}; nM)	
cellular fluorescence assays[a]	< 5000	5000 15 000	15 000 1 000 000		1 000 000 >	IC_{50} (nM)	*49*, *66*, *100*, *118*
	< 3.7	3.7 4.2	4.2 6.0		6.0 >	log(IC_{50}; nM)	

[a] Border values are defined according to published categories; the very low-affinity category was undefined.

competition experiments, whereas Table 2 summarizes assessed IC_{50} values for the peptides along with their exact amino acid sequences. All test peptides were able to inhibit at least 50% binding of the FITC-labeled reference peptide covering a spectrum of HLA A*0201 binding affinities spanning over 4 orders of magnitude, with IC_{50} values ranging from 500 to 365 000 nM. To mention is that only a partial competition curve was observed for the weak binder DLVHFASPL over the concentration range used, and therefore, the IC_{50} value is given as an approximation. No competition was detected in the case of using irrelevant unlabeled competitor peptides (data not shown).

To be in line with previously published results, we used the cell-free I^{125}-radioligand assay systems and the cellular-based fluorescence assay procedures as guidelines to define an FP-based classification system (Table 3), where peptides with an FP-based IC_{50} value of 5000 nM and lower were considered high-affinity binding, 5000−50 000 nM IC_{50} values were considered medium-affinity binding, 50 000−1 000 000 nM IC_{50} values were judged low-affinity binding, and IC_{50} values above 1 mM were regarded as no binder. Additionally, low-affinity binders were further subdivided into a low- (50 000−350 000 nM) and very low-affinity category (350 000−1 000 000 nM) because quantitative analysis of very low binders was usually less accurate and generally based on only a few data points at the upper end of the dose−response curve. According to our FP-based classification system, eight test peptides belonged to the high-affinity category; six to the medium, and one was identified as a very low binder.

Epitope Screening. Recent reports show that peptides possessing good binding characteristics are a prerequisite for successful candidate CTL epitopes (*51*, *52*). In this matter, the availability of a high-throughput peptide screening method would be practical to discover novel MHC-restricted epitopes by screening large panels of candidate peptides. To test the feasibility of such a high-throughput approach, we converted our standard competition assay procedure into a rapid screening process with the ultimate goal of identifying peptides not capable of binding to sHLA-A*0201. At a selected high threshold concentration of 80 μM, various peptides were used for this screen test including the same A2-specific peptides utilized earlier in addition to the B7-specific peptides LVMAPRTVL (HLA-B*0702 signal sequence) (*68*, *101*−*104*) and IPSYKKLIM (prostatic acid phosphatase precursor) (*105*). Experimentally, each peptide candidate (80 μM) was incubated with activated sHLA-A*0201 (50 μg/mL; 1059 nM) in the presence of 1 nM FlTC-labeled reference peptides P5 and excess β2m (2117 nM) and peptide/MHC interactions were monitored over time. Final equilibrium polarization levels were transformed in percent inhibition to indicate the extent of binding to sHLA-A*0201. Representative screening data are shown in Figure 6. As expected, all A2-specific peptides showed positive screening results, whereas both B7-specific peptides were not able to efficiently compete against the FITC-labeled reference peptide. Statistically, no significant difference among screening values for high-affinity peptides was detected, showing an average inhibition level of 97.2 ± 1.9%. This observation can be explained by the fact that high-affinity peptides reach complete inhibition below the 80 μM threshold concentration causing saturation of the system. As such, a systematic ranking of these candidate epitopes within this affinity category is not possible. However, a correct order within this group can be obtained by either screening at a lower threshold concentration or by performing a complete IC_{50} analysis as shown in Figure 5. In contrast, the rank order for medium- to low-affinity peptides was consistent with our previous results, allowing a ranking procedure without further analysis. Most important, the screening procedure clearly showed only minor to no inhibition capacity for the B7-specific peptides LVMAPRTVL and IPSYKKLIM, respectively, which provides the key for future screening applications, allowing the immediate identification of negative binders and their elimination from subsequent functional studies.

System−System Affinity Comparison. To determine whether the peptide-binding data that we have obtained is in general agreement with other studies in addition to be able to better judge the quantitative differences of IC_{50} values of our set of congeners, FP-derived binding values were compared to the peptide-binding data extracted from the literature. To

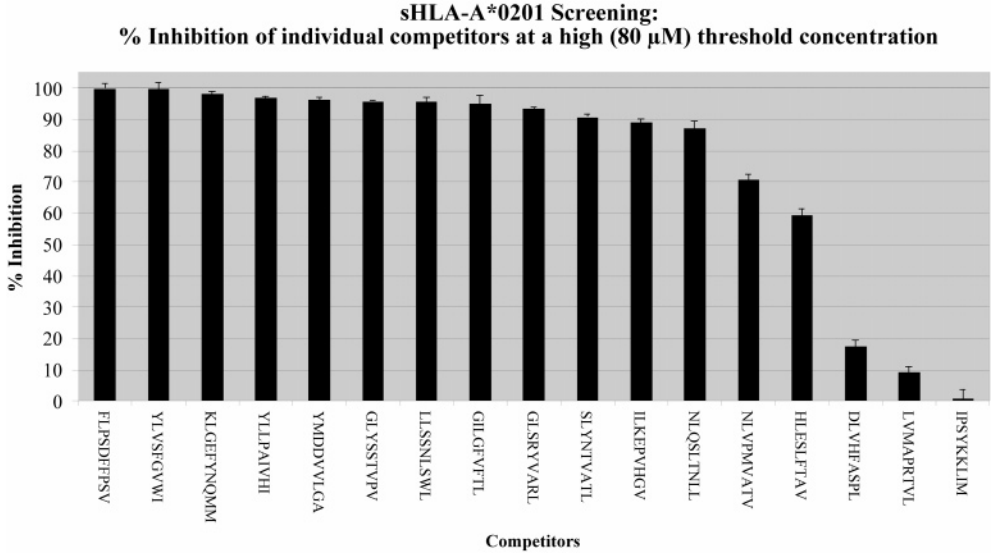

FIGURE 6: Epitope screening at a selected high threshold concentration of 80 μM. Our panel of A2-specific peptides including two negative controls related to HLA allele B7 was screened at a single threshold concentration applying our peptide competition assay for sHLA-A*0201. The unlabeled peptides (80 μM) were incubated with a constant concentration of activated sHLA-A*0201 (50 μg/mL; 1059 nM), excess β2m (2117 nM), and 1 nM FlTC-labeled reference peptides P5. After equilibrium was reached, fluorescence polarization was measured with the Analyst AD and transformed in percent inhibition relative to the control reaction without the competitor. Results demonstrate the feasibility of a high-throughput approach for epitope discovery enabling the screening of a large number of peptides with the goal to identify high-affinity binding peptides.

simplify the problem of direct comparison of absolute IC_{50} values, all affinity determinations were transformed logarithmically and plotted against each other in a log−log format. Results displayed strong linear correlations throughout the measurable range between the FP format and the cell-free I^{125}-radioligand assay system (Figure 7A) as well as between the cellular-based fluorescence assay procedure (Figure 7B). Regression analysis delivered a regression line for the cell-free I^{125}-radioligand assay system with a slope and intercept of 0.37 and 2.91, respectively. A similar linear first-order relationship was found for the cellular-based fluorescence assays with a slope of 0.55 and an intercept of 1.45, respectively. Specific outcomes were subdivided into two groups: very well-correlating peptides with an overall correlation factor 0.86 and 0.82, respectively, and poorly correlating peptides, which contributed to a much lower correlation factor of only 0.71 and 0.62. The occurrence of outliers was, however, not surprising, considering the variety of peptide-binding values collected from independent experimental procedures. Overall, the difference in performance between these particular systems seems to be only minor, considering the highly selective choice of comparative data. Conclusively, such an attribute suggests that accurate assessment of peptide binding can be obtained and the FP-based assay can be used for successful validation of unknown peptide candidates for their potential to bind sHLA-A*0201 molecules.

DISCUSSION

The identification of HLA-restricted CTL epitopes has important implications for preventive and/or therapeutic applications in infectious diseases or neoplasias. As such, a body of knowledge enhancing the ability to identify and design peptides that bind across many HLA types has emerged over the past decade, and many different methodologies have been reported with the common theme of assessing the ability of synthetically defined peptide epitopes to associate with specific HLA class-I complexes (Figure 8). A common approach for the identification of tumor antigen/virus-derived sequences recognized by CTLs consists of screening cDNA-based expression libraries (*106*, *107*) or various forms of combinatorial synthetic peptide libraries in a cytotoxicity assay format (*108*−*110*). In parallel, proteomic approaches use high-performance liquid chromatography fractionation and mass spectrometry sequencing for identification of MHC peptides eluted from normal cells of specific tissues or presented by particular MHC alleles (*68*, *87*, *101*, *111*, *112*). This nondifferential method can also be used for analysis of peptides recovered from primary tumor cells and from cells involved with pathologies other than cancer, such as autoimmune diseases and viral infections, with the aim of identifying peptides of significance for treating these diseases. More recently, mapping studies were performed with the primary intent of characterizing differences between peptide elution maps, such as between pathogenically infected versus uninfected cell lines (*113*). Differential proteomics approaches can also be used for comparisons of MHC peptide patterns induced by mutations, as a result of cancer induction or metastatic progression, induced by changes in cell-growth conditions or stress. Also very common is the utilization of computer-based algorithms, which became quit common to select immunological targets for T-cell recognition. This predictive approach basically looks for nonameric or decameric peptide sequences within a target protein, which may potentially bind to MHC class-I molecules. The matrixes are usually based on a previously determined motif and/or individual ligand sequences (*89*, *114*, *115*). Overall, these procedures are often lengthy, labor-intensive, and not free from pitfalls. Therefore, the identification of tumor- and virus-specific CTL epitopes has proceeded at a much lower rate than that of the corresponding encoding genes or proteins, and CTL epitopes derived from tumor

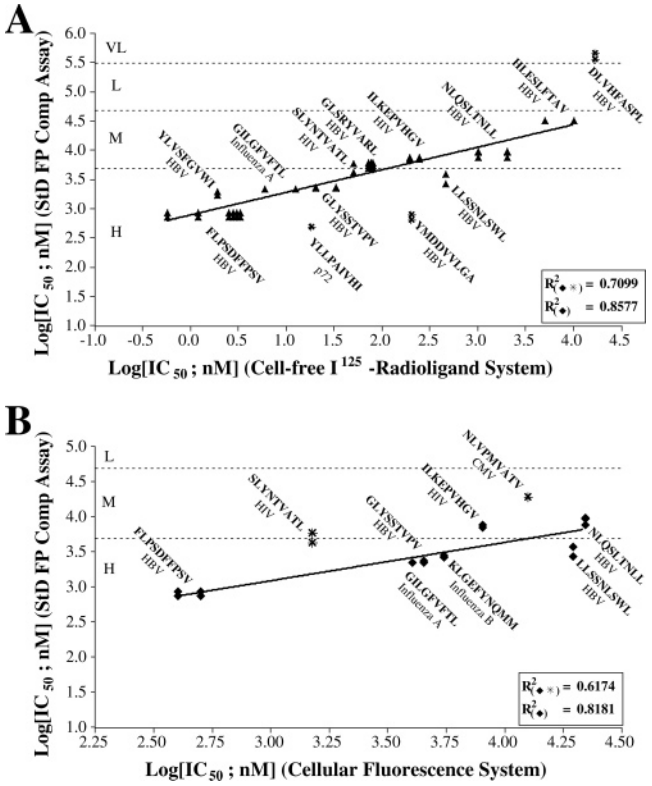

FIGURE 7: System−system affinity comparison using a variety of established peptide epitopes. The relationship between peptide-binding affinity determinations for the HLA class-I molecule A*0201 assessed through the FP system and values from (A) direct cell-free I^{125}-radioligand assay systems or (B) cellular-based fluorescence assay procedures was evaluated. Logarithmic IC_{50} values were graphed against each other, and a correlation factor was determined using linear regression analysis. Affinity values are distributed over several orders of magnitude. The classification of the peptide-binding affinity into high (H), medium (M), low (L), and very low (VL) are comparable to the classifications published by other investigators using the same reference peptides. In addition, specific outcomes were subdivided into two groups: very well-correlating peptides (◆) and poorly correlating peptides (*), contributing to much lower correlation factors.

antigens identified several years ago remain undefined. Consequently, there exists a need for novel approaches in finding validated, well-characterized MHC peptides for a protein of interest. Our biochemical approach, using FP-based peptide competition, offers a new tool set of assays, allowing epitope discovery and validation (Figure 8). Generally, competition assays measure the binding of a labeled ligand (also called reference or tracer peptide) in the presence of various concentrations of an unlabeled ligand (also called the competitor or inhibitor) to HLA. As each competitor generates its own binding isotherm, an affinity dependent IC_{50} value, the concentration of competitor necessary to displace 50% of the labeled ligand, can be obtained and directly compared to other values derived from the same system. The advantage of this approach is that, because only a reference ligand is labeled, an adverse effect on affinity potentially caused by the labeling process does not affect the comparison of the unlabeled ligands. This type of assay is not only conceptually simple but also a sensitive and most viable alternative for high-throughput applications.

A high-throughput epitope discovery process normally begins with a rapid screening for large numbers of peptides, tested at a selected high threshold concentration with the goal of eliminating peptides, which are not capable to bind to sHLA. Failing fast and cheap is highly desirable in the drug discovery world, meaning that less time and expense is wasted on compounds that would not have passed the next-stage hurdles because of the lack of binding. As seen in Figure 6, our newly developed epitope screening approach is highly flexible to identify peptides from any protein of interest, making this approach a key factor in early epitope discovery. The measurements provide data of very high precision and reproducibility.

High-affinity binding is thought to be a critical factor controlling immunogenicity of peptides (*98*, *116*). Within the epitope discovery process, characterization of peptide and MHC interactions can be achieved using the same plate-based assay format with slightly different assay conditions. This validation approach enables the determination of the inhibitory concentration IC_{50} on positively identified peptide candidates (Figure 5), with the goal to optimize data quality by higher resolution analysis and also to deliver comprehensive information for assay development stages (Figures 1−3). A common approach is the ranking of identified peptides according to their immunogenic potential with the assumption that high-affinity binding peptides are preferred over low-affinity candidates. However, definitions of target identification vary. In general, identification falls along a spectrum ranging from simply cataloging a molecule's existence to ascertaining a therapeutically relevant function in cell and animal models. Identification always comes back to unbiased searches, those that do not skew results toward a certain subset of targets. The quality of anyone data set is such that you can never choose the top one or two and be confident that the selection criteria have actually led you to the one or two best candidates. Therefore, any one of these candidates has generally the potential to be successful as vaccines. In addition, the IC_{50} determination approach is also used to evaluate binding results achieved after introduction of sequence alterations thought to improve the potency of the peptide.

The FP-based technology provides a range of additional approaches to further analyze and validate epitope targets such as the characterization of the fluorescent-labeled version of the peptide of interest (*86*), allowing real-time kinetic measurements and determination of equilibrium dissociation constants (K_d). Furthermore, with the availability of more than 50 of the most common sHLA alleles, direct experimental detection of overlapping peptide-binding capacities among this large set of alleles became feasible, reflecting the ability of MHC class-I alleles with genetically more or less distinct peptide-binding sites to share the binding of identical peptides (*86*). Classification of allele overlapping peptide-binding specificities may become an important issue in vaccine design, with direct implications concerning population coverage. Dependent upon individual allelic composition, it is import to identify the binding capacity of single peptides to determine their usefulness for treating a larger subset of patients who express the MHC alleles that are capable of binding that specific peptide.

Overall, several lines of evidence, both at the biological and functional level, emphasize the biologic relevance of peptide-binding assays in the identification and evaluation of potential peptide candidates (*50*−*53*). Our binding assays comprise a number of significant advantages compared to

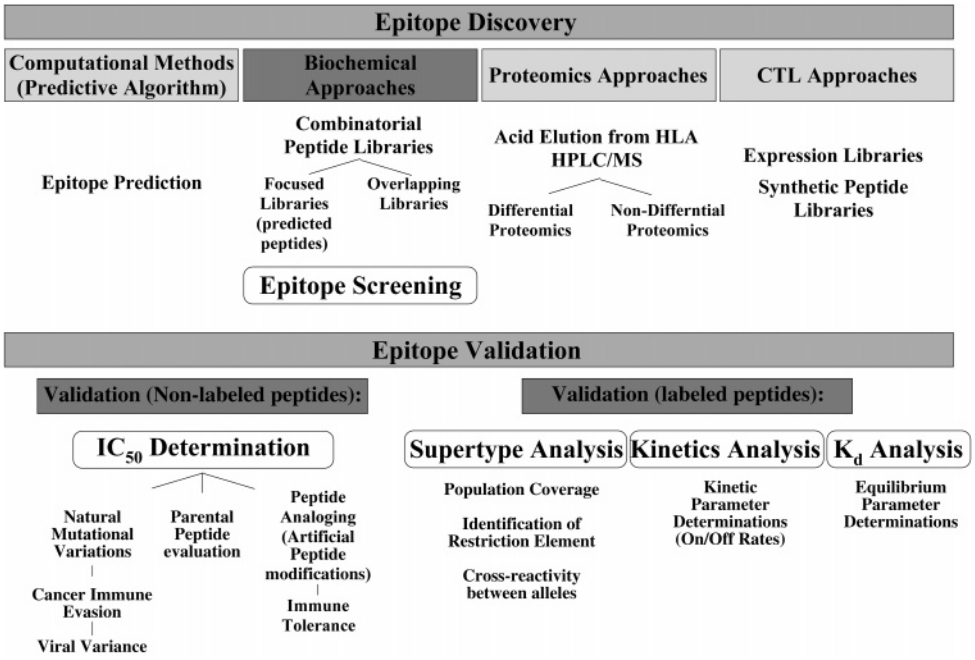

FIGURE 8: Epitope discovery and validation tools. The upper part of the scheme summarizes various methods currently applied in CTL-epitope discovery. The lower part shows approaches feasible for epitope validation using FP-based technologies, which are subdivided into systems using nonlabeled versus labeled peptides as validation objects. Methodologies, which use standardized FP-based peptide-binding assays for analysis, are circled.

existing other HLA class-I binding assays. The most important features are the excellent reproducibility and high sensitivity. Good reproducibility is a key parameter in screening extensive sets of peptides for their affinity to HLA, and crucial information is derived from comparisons of various peptides. Furthermore, FP is unique among methods used to analyze molecular binding because it gives a direct, nearly instantaneous measurement of a peptide tracer's bound/free ratio in solution. A truly homogeneous technique, which does not require the separation of bound and free species, makes the assay a very simple one-step procedure, which is particularly appropriate for high-throughput screening where all reagents remain in solution. Such assays are easily automated and have the ability to rapidly screen whole libraries for peptide candidates. Methods that depend on separation are not only more time-consuming, but they also disturb the reaction equilibrium and therefore prevent accurate quantification of binding. In addition, it is worthwhile to note that the assay is nonradioactive and can thus be performed in virtually any laboratory without the risk of radioactive contamination.

A common practical problem encountered experimentally in a variety of competitive binding studies is the lack of standardization, seldomly allowing direct comparison of IC_{50} values between different assay approaches. As visualized in Tables 2 and 3, differences for IC_{50} determinations between chosen systems range from 2- to 900-fold when comparing FP data to the I^{125}-radioligand assay systems and 0.2- to 5-fold when compared to the cellular-based fluorescence assay systems. As such, it seems apparent that the task of IC_{50} determinations by competitive ligand-binding studies requires a more standardized approach than has been initially considered adequate, justifying the further development of such studies, particularly in view of identifying new potential epitopes for vaccine development. Therefore, as a first step toward a more robust approach, we attempted to better assess the factors influencing competition binding curves and consequently IC_{50} values. One of the most critical factors found to affect IC_{50} values is the HLA molecule itself, which was identified as generally the most variable assay component. The usage of HLA derived from homozygous cell lines, as lysate or intact cells, is common practice in competition assay studies (*56*, *66*, *79*, *82*, *83*). However, the problem of establishing suitable models and reliable data for MHC class-I/peptide interactions is vastly complicated when a heterogeneity of alleles is present. In some cases, antibodies against an individual allele are available to achieve more specificity but required specialized purification procedures, which are cumbersome, time-consuming, and usually do not deliver sufficient quantities for experiments in a large scale. Another factor that influences the efficiency and reproducibility in studies utilizing HLA-expressing cell lines is the level of class-I expression that generally varies from cell line to cell line. Because the functional response to a ligand is related to receptor density, this variability can lead to a rather wide range of variation, which is not suitable for reliable data comparison. Therefore, the most important advantage of utilizing recombinant sHLA molecules produced in cell lines that do not spontaneously express HLA ("null" cell lines) resides in the capacity to standardize the amount of sHLA employing sHLA preparations of defined specificity and purity, as well as exhibit excellent stability on storage.

When we focused on standardization issues, a striking example of the effect of sHLA concentration on the IC_{50} values of unlabeled peptides is shown in Figure 3. It is evident that increasing the concentration of sHLA markedly increased the concentration of unlabeled competitor required to inhibit 50% of the binding of FITC-labeled reference peptide resulting in an underestimation of the potency of the displacing ligand. Therefore, to achieve experimental accuracy and reproducibility, it is necessary to work with a standardized quantity of sHLA molecules. The importance

of receptor concentration in determining relative potencies of competitive inhibitors has been described earlier (*91*). Furthermore, it was demonstrated that direct binding studies of ligand−receptor interactions of high affinity are subject to artifactual distortions because of the need to utilize high concentrations of the receptor. Therefore, it would be advantageous to design competition experiments such that the [sHLA]/K_d ratio is much lower than 0.1. However, such conditions do not seem experimentally approachable for FP-based peptide-binding experiments, because relatively high concentrations of sHLA receptors are needed to yield a significant change in polarization. Consequently, more inhibitor is required to see a 50% drop in the amount of tracer ligand during competition. The result is that the observed IC_{50} values are an overestimation of the true dissociation constant for the unlabeled ligand (K_i). Overall, the FP system is affected by severe competitor depletion (*90*), explaining why IC_{50} values are several orders of magnitude higher than their K_i values, an effect also seen in other assay systems (*49*, *66*, *82*). Because of this depletion effect, the historically used method of Cheng and Prusoff (*117*), transforming IC_{50} values into K_i values, is not applicable because the necessary substitution of total concentrations for free concentrations cannot be made without introducing significant errors in the calculation of K_i. In addition, the presence of unknown amounts of endogenous, class-I-associated peptides that could potentially compete with the tracer and test peptide during the course of the assay would even further complicate such mathematical transformations. Nevertheless, results derived from dose−response curves remain extremely useful in comparing relative affinities of ligands to the HLA receptor and in determining whether the ligand of interest in a series of compounds can cause the same maximal response (indicative of a closely similar interaction with the recognition site) as other ligands.

In addition to the effect of the HLA concentration, variations in the choice of the tracer peptide and the inconsistent usage of tracer concentrations are also contributing factors for inconsistent data output. The impact of different reference peptides on IC_{50} values shown in Figure 1 clearly indicates that the development of a standardized assay necessitates the consistent usage of the same reference peptide to deliver reproducible results. As such, a critical initial step toward development of any sHLA assay is the choice of a single FITC-labeled peptide candidate able to specifically bind to the allele of interest. Because of the specific nature of competition assays, the selection process for a suitable tracer involves considerations relating more to the chemical rather than the biological nature of the candidate. Because pFITC-labeled reference peptides are not under investigation in these assays, maintaining the native reactivity of the candidate reference peptides was not necessary. Therefore, to achieve most optimal assay performance qualities, the highest priorities are normally given to features such as stability and affinity of the design, as well as dynamic range of the polarization signal allowing sensitive detection of a small decrease in polarization upon the addition of a competitor peptide. Extensive analysis showed that the pFITC ligand ALMDKVL-K(FITC)-V (P5), an artificially designed peptide whose primary sequence was derived from sequence pools of naturally processed peptides, best met the requirements for an optimal A*0201 tracer candidate (*86*) by showing a very high stability and a unique dynamic range of over 215 mP.

Furthermore, an optimal pFITC reference concentration had to be selected, to ascertain sensitivity but also guaranteeing maximal dynamic range. Unlike classical binding curve profiles, FP signals are greater for low ligand concentration because both the bound and free fluorescent ligand contribute to the final signal. Consequently, to achieve maximal change in polarization, it is necessary to use low fluorescent ligand concentrations because increasing pFITC concentrations would require increasing "free" sHLA to ensure that the same fraction of ligand is bound. Therefore, a low concentration of 1 nM FITC-labeled peptide was chosen, which enables a high ratio between maximum and minimum FP signals and also displays low standard deviation values. To control the accuracy of all fluorescent peptide concentrations used within this study, concentration values were normalized by comparing the molar fluorescence intensity of each fluorophore-labeled peptide with the FITC fluorophore molecule itself. The importance of this task should not be underestimated, because inaccurate assignment of concentrations can cause high IC_{50} fluctuations. As shown in Figure 2, applying higher pFITC concentrations took larger concentrations of unlabeled peptide to compete for half of the binding sites and therefore increased the IC_{50} values. According to the steepness of the regression line, it became obvious that a ±1 nM variance in concentration will cause a ±627 nM variance in the IC_{50} value.

Another important step of our study besides standardization was to further explore the binding specificity of the HLA A*0201-restricted FP-based competition assay. For this reason, a panel of well-defined HLA class-I ligands from various sources covering a broad range of binding affinities was tested (Table 2). These peptides were almost all of viral origin except for a single peptide derived from an RNA-dependent helicase p72. Results showed that, in all cases, the previously known A*0201 specificity was confirmed. More importantly, the assay was capable of measuring IC_{50} values over a range of 4 orders of magnitude (from 500 to 365 000 nM), quantitatively characterizing the binding strength of various existing sequences. Because dose−response curves are unable to confirm or disprove any mode of interaction of peptides with sHLA, they are only useful in comparing relative affinities of ligands with the HLA receptor as well as determining whether the ligand of interest in a series of compounds can cause the same maximal response (indicative of a closely similar interaction with the recognition site) as other peptides. Therefore, a common approach for peptide evaluation is the quantitative ranking of identified peptides according to arbitrarily defined categories along a spectrum of high-, medium-, and low-affinity binding to ascertain a therapeutically relevant function in cell and animal models. Built upon existing models earlier defined by Sette et al. (*34*, *48*, *56*, *81*, *94*, *99*) and van der Burg et al. (*49*, *66*, *82*, *100*, *118*), an FP-based IC_{50} value classification scheme was created (Table 3). When we focused on the correlation between peptide-binding affinity and immunogenicity, all control peptides selected with known CTL activity showed, as expected, a high to medium-high affinity to sHLA-A*0201. Among our viral controls, the HBV-derived epitope FLPSDFFPSV was found to display very high-affinity values. As one of the most

referenced peptides found in the literature, FLPSDFFPSV is known for high-affinity binding to A*0201 (*34*, *56*, *81*, *93*) as well as the ability to induce potent and specific CTL responses (*119*). Additional members of the high-affinity category were the peptides SLYNTVATL, another well-studied HIV-derived CTL epitope (*120*−*122*), and the influenza matrix peptide GILGFVFTL, found to be a major target of influenza-specific CTL, both in humans (*123*−*129*) and in HLA-A2 transgenic mice (*130*).

As final step in validating our FP system, IC_{50} determinations were compared to the two quantitative assay systems most commonly referred to in the literature, the direct cell-free I^{125}-radioligand assay (*40*, *48*, *51*, *56*, *94*−*99*) and the cellular-based fluorescence assay (*49*, *82*, *100*). A significant correlation between the logarithm of the IC_{50} values from our FP-based system and values from the two reference systems was found by linear regression analysis (Figure 7). During analysis, some outlier values were noted for both systems, a discrepancy certainly expected considering the selection of affinity data from independent assay sources. As such, single differences found in affinity might rather be due to the lack of normalization in obtaining binding values as discussed above, which resembles a current problem in analysis of competition data between individual assay systems. In addition, the solubility of the peptide as well as the challenge of accurate weighing of solid peptides may further contribute to the inaccurate determination of binding values. Particularly, dissolving peptides is a critical step because the wrong choice of solvents and methodology can lead to inaccurate or complete lack of performance. As described earlier within the text, the usage of DMSO or DMF, which are widely accepted in many end-use applications for peptides, seems most appropriate for biochemical peptide-binding assay procedures. Nevertheless, results obtained clearly suggest that accurate assessment of peptide binding can be obtained by using our novel FP-based assay procedure.

In summary, our standardized FP-based peptide-binding assay provides uniquely efficient means of identifying and evaluating immune target molecules that are recognized by cytotoxic T cells. The wealth of newly available genomic sequence information provides a superb source for the identification of novel targets to use as the basis of the design of new specific and selective therapeutic agents and diagnostics. The concept of our assays can be adapted for basically every HLA class-I allele of interest. Although the data presented here were generated using sHLA-A*0201, additional studies indicate that this approach works equally well with other sHLA alleles. Numerous significant MHC-restricted epitopes have already been defined for B*0702 (*113*), and more studies are on the way. Therefore, the present report can also be read as an instruction for the development of class-I binding assays that are still lacking. This assay's capacity for high-throughput evaluation of peptide/HLA interactions, coupled with the ability of the immune system to recognize virus-infected or cancerous cells, will open new possibilities to identify an ever-burgeoning number of immunologically active peptide epitopes for the development of vaccines to treat or prevent various types of infectious diseases or cancers. Furthermore, the FP assay has great potential to provide new database information and help to increase missing knowledge, which can be utilized for the development of more sophisticated algorithms to predict and quantify the binding affinity to HLA class-I molecules.

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