Real-Time Measurement of in Vitro Peptide Binding to Soluble HLA-A*0201 by Fluorescence Polarization

Rico Buchli,*[,‡,§] Rodney S. VanGundy,[‡] Heather D. Hickman-Miller,[§] Christopher F. Giberson,[‡] Wilfried Bardet,[§] and William H. Hildebrand[‡,§]

Pure Protein L.L.C., Oklahoma City, Oklahoma 73104, and Department of Microbiology and Immunology, Health Sciences Center, University of Oklahoma, Oklahoma City, Oklahoma 73104

Received July 5, 2004; Revised Manuscript Received September 13, 2004

ABSTRACT: Measuring the interaction of class I human leukocyte antigens (HLA) and their peptide epitopes acts as a guide for the development of vaccines, diagnostics, and immune-based therapies. Here, we report the development of a sensitive biochemical assay that relies upon fluorescence polarization to indicate peptide interactions with recombinant soluble HLA proteins. It is a cell- and radioisotope-free assay that has the advantage of allowing the direct, real-time measurement of the ratio between free and bound peptide ligand in solution without separation steps. Peptide/HLA assay parameters were established using several HLA A*0201-specific fluorescein isothiocyanate-labeled peptides. Optimal loading of synthetic peptides into fully assembled soluble HLA-A*0201 complexes was enabled by thermal destabilization at 53 °C for 15 min, demonstrating that efficient peptide exchange does not require the removal of endogenous peptides from the reaction environment. An optimal ratio of three β-2 microglobulin molecules per single HLA heavy chain was determined to maximize peptide binding. Kinetic binding studies indicate that soluble HLA-A*0201/peptide interactions are characterized by a range of moderate k_{on} values (1×10^4 to 8.7×10^4 M^{-1} s^{-1}) and slow k_{off} values (1.9×10^{-4} to 4.3×10^{-4} s^{-1}), consistent with parameters for native HLA molecules. Testing of the A*0201-specific peptides with 48 additional class I molecules demonstrates that the unique peptide binding behavior of individual HLA molecules is maintained in the assay. This assay therefore represents a versatile tool for characterizing the binding of peptide epitopes during the development of class I HLA-based vaccines and immune therapies.

The determination of in vitro equilibrium and kinetic data for specific peptide/HLA[1] class I interactions is a primary means of identifying viral, bacterial, and cancer vaccine candidates. Many different methodologies have been reported with the common theme of assessing the ability of synthetically defined peptide epitopes to associate with specific class I complexes. A common cellular method is the cell surface stabilization assay, demonstrating the assembly of empty class I HLA molecules on the surface of TAP-deficient T2 cells by exogenously adding iodinated or fluorescent-labeled peptides (*1*, *2*). Another cellular approach involves the mild acid treatment of HLA-expressing cell lines (*3*−*6*), some transfected to overexpress human HLA (*7*−*9*), which has been used as an alternative method to generate empty class I molecules. Instead of using intact cells, traditional biochemical binding assays utilize detergent-solubilized HLA class I complex molecules in combination with iodinated peptides to measure peptide binding (*10*−*15*). In these conventional radioactive binding assays, radioactive iodine (^{125}I) is measured after chromatographic separation of bound from free peptide ligand (*12*, *15*, *16*). Furthermore, de novo reconstitution assays are also very common, incubating synthetic peptides with free HLA heavy chains and free β-2 microglobulin (β2m) to assemble class I from its subunits and comparing the resultant quantities of free versus complexed heavy chains (*17*−*19*). Presently, these assays have been established in only a few laboratories and are usually time-consuming and extremely laborious. A further limitation in many of these heterogeneous binding assay applications has been the lack of availability of class I HLA proteins. Our laboratory has developed a means for producing substantial quantities of numerous soluble HLA (sHLA) alleles (*20*, *21*). It was intended to utilize these sHLA molecules for the development of a more robust peptide/HLA binding assay.

Several fluorescence-based assay formats are now in development to determine kinetic constants including fluorescence resonance energy transfer (FRET) and homogeneous time-resolved fluorescence (HTRF). In addition, surface plasmon resonance (SPR), a technique based on immobilization of one of the interacting components to a biosensor

* To whom correspondence should be addressed at Pure Protein L.L.C., 800 Research Parkway, Suite 340, Oklahoma City, OK 73104-3698. Tel: 405-271-3838. Fax: 405-271-3848. E-mail: rico-buchli@ouhsc.edu.

‡ Pure Protein L.L.C.

§ University of Oklahoma.

[1] Abbreviations: HLA, human leukocyte antigen; sHLA, soluble HLA; β2m, β-2 microglobulin; TAP, transporter associated with antigen processing; FRET, fluorescence resonance energy transfer; HTRF, homogeneous time-resolved fluorescence; SPR, surface plasmon resonance; FP, fluorescence polarization; mP, millipolarization; FITC, fluorescein isothiocyanate; pFITC, FITC-labeled peptide; DMSO, dimethyl sulfoxide; BGG, bovine γ-globulin; BSA, bovine serum albumin; PCR, polymerase chain reaction; FCS, fetal calf serum; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CTL, cytotoxic T lymphocyte; k_{ob}, observed rate constant; k_{on}, association rate constant; k_{off}, dissociation rate constant; K_d, equilibrium dissociation constant.

10.1021/bi048580q CCC: $27.50
Published on Web 10/30/2004

Table 1: FITC-Labeled Peptide Sequences[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/P3	N	L	S	**K**	L	S	L	D	V	
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] Amino acid positions carrying the FITC label are in bold type.

surface (*13*), has also gained some popularity. Among these advanced assays belongs the technique of fluorescence polarization (FP), a powerful but underutilized tool for studying molecular binding interactions. First described in 1926 (*22*), FP is unique among methods used to analyze molecular binding events because it gives a direct, nearly instantaneous measure 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 (*23*, *24*), resulting in an increase in polarization.

Given the need for quantitative and widely applicable peptide/HLA binding assays, we recognized the value of FP-based binding analysis. However, to experimentally evaluate polarization, two measurements are needed: polarized emission parallel to the excitation filter (S-plane) and polarized emission perpendicular to the excitation filter (P-plane). Resulting FP values in millipolarization (mP) are calculated by the equation

$$\text{polarization (mP)} = (S - GP)/(S + GP) \times 1000$$

where S and P are background-subtracted intensities of the fluorescence measured in the parallel (horizontal) and perpendicular (vertical) direction, respectively, and G (grating) is an instrument- and assay-dependent factor.

In this report, we describe the co-incubation of purified, recombinant sHLA molecules with FITC-labeled peptides that have been previously reported to have a high affinity for A*0201 as unlabeled peptides. Allele A*0201 was chosen because it is found at a high frequency in the population and is the most extensively studied HLA class I antigen. The resulting data demonstrate that an FP-based HLA/peptide binding assay is feasible, quantitative, and does not depend on radioactivity. Because of the real-time nature of the assay, kinetic measurements are readily obtainable. By testing the A*0201-specific peptide ligands against various class I, we demonstrate that individual sHLA molecules maintain their peptide binding behavior using the described method. This assay is relatively simple to conduct, can be standardized to different FP readers, and is amenable to the unbiased study of many different peptide/HLA interactions.

MATERIALS AND METHODS

Synthetic Peptides. FITC-labeled peptides (Table 1) were commercially synthesized by Synpep (Dublin, CA) or American Peptides (Sunnyvale, CA) using solid-phase strategies and purified with reverse-phase HPLC. Purity was determined to be greater than 95%. The composition was ascertained by mass spectrometric analysis. Peptides were ordered in aliquots of 0.2 μmol amounts and stored dry. Lyophilized peptide aliquots used in binding assays were originally dissolved in 100% DMSO at a concentration of 10 mM. Subsequent dilutions were done in 1× bovine γ-globulin in PBS (BGG/PBS, 0.5 mg/mL, 0.05%; Sigma, St. Louis, MO). Aliquoted working dilutions (200 μM) for FITC-labeled peptides were kept at −20 °C and reused for a period of up to 4 months without a measurable decline in signal.

Protein Expression and Large-Scale Production of Recombinant sHLA Class I Molecules. All sHLA molecules used within this study were cloned and expressed according to the techniques described previously (*20*). Briefly, truncating PCRs were performed using specific primer sets and template DNA from reliable full-length cDNA clones of the HLA alleles of interest. The resultant PCR products contained the leader peptide, as well as the α1, α2, and α3 coding domains of the HLA heavy chain resulting in the removal of the portion encoding the membrane-spanning region and the cytoplasmic tail. PCR products were directly subcloned into the mammalian expression vector pcDNA3.1(−) (Invitrogen; Carlsbad, CA) and sequenced to confirm allelic specificity. The class I negative, EBV-transformed B-lymphoblastoid cell line 721.221 was transfected with a cloned DNA construct by electroporation, and stable transfectants were selected using 1.5 mg/mL G_{418}. Upon establishment of confluent growth after approximately 3 weeks, putative transfectant wells were screened for sHLA production using a sandwich ELISA (*20*). Transfectant wells positive for sHLA production were then subcloned by limiting dilution. Satisfactorily subcloned transfectants were expanded, frozen in RPMI-1640, 20% FCS, and 10% DMSO, and stored at −135 °C.

After high-level producers were established, the CP-2500 Cell Pharm System (Biovest International, Minneapolis, MN) was applied in order to produce large quantities of sHLA (*20*, *21*). Transfected 721.221 cells were first expanded over a time period of approximately 21 days before seeding two hollow-fiber bioreactor units of the Cell Pharm. 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 crude harvest.

Affinity Purification of sHLA Molecules. Upon completion of a bioreactor run, sHLA complexes were affinity purified from the harvests obtained using the pan-HLA class I antibody W6/32 (*25*) coupled to a Sepharose 4B matrix (Amersham, Piscataway, NJ). Harvests were applied to the column using a peristaltic pump system (Amersham) with a speed of 5 mL/min at 4 °C. After the column was extensively washed with phosphate-buffered saline (PBS), bound sHLA molecules were eluted with 0.1 M glycine (pH 11.0) and immediately neutralized by addition of 1 M Tris-HCl, pH 7.0, to preserve the activity of the eluted molecules. Under these conditions, sHLA molecules were recovered with a 75−85% yield. 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 by the colorimetric detection method using the Micro BCA protein assay kit (Pierce, Rockford, IL) using BGG as

a standard. Gel electrophoresis−sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed, which confirmed the size of the subunits and their purity (data not shown).

ELISA-Based Stability Testing. For stability evaluation, a specific HLA sandwich ELISA approach was adopted as described earlier (*20*) with minor modifications. Briefly, to detect the antigen sHLA-A*0201, the wells of a microtiter plate were coated with the specific (capture) antibody W6/32 (8 μg/mL) followed by the incubation with test solutions containing the antigen. Unbound antigen was then washed away, and a second antigen-specific antibody (anti-β2m) (DAKO, Glostrup, DK) conjugated to HRP (detector) was added. After addition of the HRP substrate OPD, the degree of substrate hydrolysis was measured. ELISA data were finally transformed to percent relative stability using the nonheated solution of sHLA-A*0201 (4 °C) as reference (100%). Data are graphed against hours of incubation, and the half-lives were determined by applying a one-phase exponential decay model.

FP-Based Peptide Binding Assays. In a first step, the three components of the binding reaction (peptide, sHLA, and β2m) were prepared as concentrates. The fluorescent-labeled peptide (pFITC), listed in Table 1, and the sHLA component of the reaction were diluted to appropriate 4× and 2× solutions, respectively. The β2m component (Fitzgerald Industries International, Concord, MA) was prepared as a 4× mix and always added in a 2× molar excess of the used sHLA concentration. For all preparations, 1× BGG/PBS was used as buffer. In a second step, each individual well of a black 96-well LJL HE PS microplate (Molecular Devices) was loaded with 10 μL of the prepared 4× β2m and 10 μL of 4× pFITC. To start the peptide exchange procedure, the 2× sHLA mix was activated by incubation at 53 °C for 15 min before addition of 20 μL to the previously loaded wells, reaching a final volume of 40 μL. All reagents were added to the wells of the microtiter plate sequentially using manual pipettors. Immediately after addition of all fluids, the plate was spun down for 1 min at 2500 rpm to even the meniscus and remove possible air bubbles. The plates were incubated at room temperature 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.

The use of appropriate controls allowed accurate estimation of specific polarization. Specific control groups included (a) protein only, (b) tracer only, and (c) buffer only. The protein only control indicated the background signal contributed from sources other than the fluorescent peptide, which was eliminated by subtraction from the experimental sample. For proper analysis, *S* (parallel) and *P* (perpendicular) intensities were background corrected. Background signals can arise from the microplate plastic, solution contaminants, leakage of light through the optical filters, or other sources generated in the instrument. Background-subtracted with the buffer only control, the tracer only control measured *S* and *P* values for free fluorescent peptide. As described by Herron and Voss (*26*) and according to the instructions of the manufacturer of the Analyst AD, these corrected values serve in the calculation of the *G* factor [G factor $= I_S/I_P(1 - (27/1000))/(1 + (27/1000))$], which is a scaling (correction) factor, taking relative polarization measurements and making them appear absolute (relative to the theoretical mP (27 for fluorescein). Within this study, *G* factors were very stable values typically ranging from 0.9 to 1.1. Once a *G* factor had been determined, it was entered into the formula to calculate polarization: $\mathrm{mP} = (I_S - I_P G)/(I_S + I_P G) \times 1000$.

FP measurements were performed on the Analyst AD assay detection system (Molecular Devices, Sunnyvale, CA) using a continuous high-intensity, xenon-arc lamp as light source with the following filter settings: excitation wavelength of 485 nm and emission wavelength of 530 nm. In a standard FP configuration, the static excitation polarization filter was set in S position whereas the dynamic emission polarization filter polarized the light in either the S or P orientation. A dichroic mirror (50/50 beam splitter) 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). The two intensity measurements collected for each well, one when the polarizers were parallel to each other (S and S) and one when the polarizers were perpendicular to each other (S and P), were used to calculate polarization in millipolarization units (mP).

Time-Course Analysis of sHLA/Peptide Association. Peptide kinetics of the ternary complex formation was investigated by monitoring the association of FITC-labeled peptides to sHLA molecules by FP, which allows direct measurement of the time course of the reaction. To determine the association kinetics, the purified sHLA molecules (50 μg/mL) and various concentrations of fluorescent-labeled peptides were incubated in the presence of excess β2m (24.8 μg/mL) in a final volume of 40 μL. Changes in FP were monitored starting shortly after addition of the activated sHLA at time 0, until equilibrium was reached. Reactions were setup directly in 96-well black plates. Measurements were performed on the Analyst AD as described above.

Binding parameters were achieved by fitting all data points to a monoexponential association model [$\mathrm{FP} = \mathrm{FP}_{max}(1 - e^{-kt})$]. FP is binding and t is time. The variable k in the exponential association equation is the observed rate constant (k_{ob}), expressed in units of inverse time, which is concentration dependent. To calculate absolute rate constants for association (k_{on}) and dissociation (k_{off}), k_{ob} values were graphed versus the ligand concentration. Extrapolation of the plot to zero ligand determined the intercept, which is equal to k_{off}. The slope of the graph corresponded to k_{on}.

RESULTS

State-of-the-art biochemical assays are comprised of technologically advanced platforms and cutting edge reagents. Such assays must also be practical in many laboratory environments and, in this case, amenable to many different peptide/HLA combinations. Here, we developed a FP-based system for real-time peptide binding analysis with the overall objective of providing a robust and highly sensitive assay for the better characterization of HLA/peptide interactions.

FITC Labeling of Synthetic Peptide Candidates. The initial step toward assay development was the selection of high-affinity peptide candidates and the attachment of a fluorescein isothiocyanate (FITC) label to these peptide candidates. Because the assay represents a quantitative characterization

of FITC-labeled peptides, we strove to maintain insofar as possible the native reactivity of the candidate molecules. This goal implies that the functional groups involved when the peptide reacts with the sHLA molecule remain fully active and unimpeded and that the fluorescent label itself does not become involved in, or perturb, the reaction. The chemical addition of a FITC molecule to a peptide requires distinct amino acid residues, and only side chains containing primary amines (lysines, K) or sulfhydryl groups (cysteins, C) can be fluorochrome-labeled. Therefore, the fluorescent labeling of peptide candidates makes it mandatory to perform amino acid substitutions if these amino acids are absent within the primary structure of the peptide candidate. However, substitution of amino acids is not without risks as these residues can exert important effects on the binding capacity of a peptide. Therefore, considering only sequence positions with known permissiveness (*14*), positions P4, P5, and P8 for 9-mers and positions P5 and P6 for 10-mers were selected to allow the chemical coupling to the primary amine of a lysine side chain (Table 1). Important to mention is that labeling of an internal K residue for A*0201 peptides is not considered a conservative substitution. Such a modification will likely affect the interaction in a negative way as the FITC-coupling reaction changes the positively charged residue to a hydrophobic group, thus losing its capability for electrostatic interaction.

Peptide Exchange Procedure. Since the recombinant sHLA class I proteins used for this study are loaded with endogenous peptides (*11*), binding of FITC-labeled peptides can only occur through a peptide exchange procedure in which the original endogenous peptide is first released from the HLA binding groove. Therefore, our second but most challenging step in the development of the A*0201 peptide binding assay was the design of an effective loading procedure for FITC-labeled peptides. Because difficulties of loading native HLA molecules with synthetic peptides are well-known, several protocols have been designed. One of the simplest methods described in the literature was passive loading in which the peptide exchange is observed at room temperature. However, initial testing of multiple sHLA concentrations incubated with pFITC P2 demonstrated that passive loading did not work particularly well (Figure 1A). The measured change in polarization values upon binding under equilibrium conditions was only 40 mP, reaching a maximal polarization level of 66 mP for the highest concentration used. These results corroborate with earlier findings showing experimental difficulties in demonstrating peptide binding to complexes that contain endogenous peptides (*27*). The low loading efficiency was attributed to the stability of the endogenous peptide/class I molecule complex. Other methods described included the stripping of peptides through alkaline or mild acid treatment followed by gel filtration and slow refolding at neutral pH to obtain HLA class I heavy chains without the presence of the endogenous peptides or β2m (*28*−*31*). However, the heavy chains prepared through these procedures were found to be unstable and only useable for a short amount of time (*28*), which makes such stripping procedures ill-suited for more advanced and standardized binding assays as stability has a direct impact on assay quality and reproducibility. Since most effective loading seems to be attained only when empty class I proteins become available (*1, 32*), we developed a novel

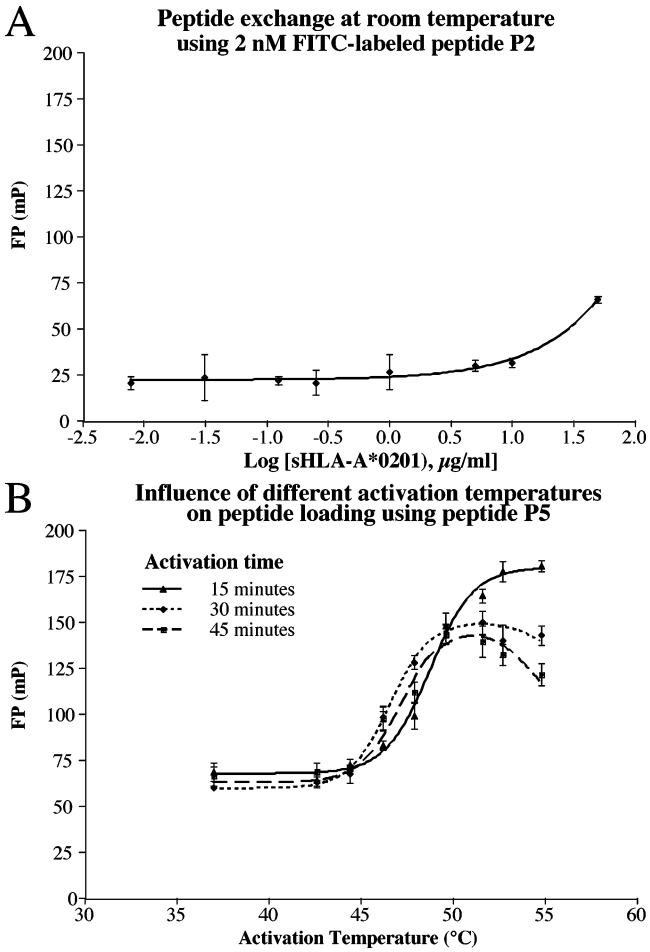

FIGURE 1: Peptide exchange analysis. (A) Peptide exchange was monitored at room temperature over a sHLA concentration range of 0.01−50 μg/mL. Results demonstrate a low signal output making passive loading highly inefficient. (B) The influence of different activation temperatures on peptide exchange efficiency was tested by activating 50 μg/mL sHLA followed by incubation with 10 nM pFITC P5. Most optimal conditions were established using an activation temperature of 53 °C for 15 min to achieve maximal polarization levels. Longer activation times are shown to be suboptimal for highest loading efficiency.

approach to increase the fraction of empty, peptide-receptive class I molecules. Our approach was based on the idea that peptide loading efficiency can be improved through the activation of sHLA molecules by thermal energy. Incubating sHLA samples temporarily at higher temperatures should thermodynamically destabilize the sHLA complex, allowing more peptide-receptive sHLA molecules to participate in the peptide exchange. However, the extent of energy employed has to be carefully controlled to ensure that the majority of the molecules are capable of restoring proper conformation after the reinstatement of original conditions.

As seen in Figure 1B, we tested sHLA activation as a function of temperature and time to identify optimal peptide loading conditions. Identical sHLA samples (50 μg/mL) were incubated at various temperatures over a period of 15, 30, and 45 min. After activation, the samples were immediately combined with 10 nM pFITC P5 to initiate the experiment. The association of labeled peptides was monitored in real time to ensure that equilibrium was reached. Results obtained showed a steady increase in polarization with higher activation temperatures. Short-term activation for 15 min was found

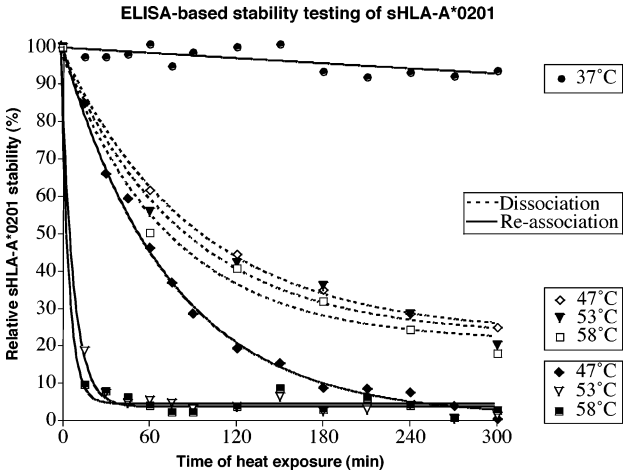

FIGURE 2: ELISA-based sHLA-A*0201 stability determination using conformational epitope-recognizing antibody W6/32. An assay procedure consisting of the monoclonal antibody W6/32 as the capturing reagent and anti-β2m(HRP) as the detector antibody was developed to test sHLA-A*0201 complex stability at various temperatures during a period of 5 h in 3% BSA. After incubation, the solutions were immediately tested on ELISA, preventing re-formation of destructed/destabilized molecules. ELISA data are transformed to % relative stability using a sample held at 4 °C as 100%. Data are graphed against time of heat exposure and half-lives determined by applying a one-phase exponential decay model (solid lines). In addition, a selection of heated samples were reevaluated after a 72 h incubation time at 4 °C to determine complex re-formation (dashed lines) and added to the graph.

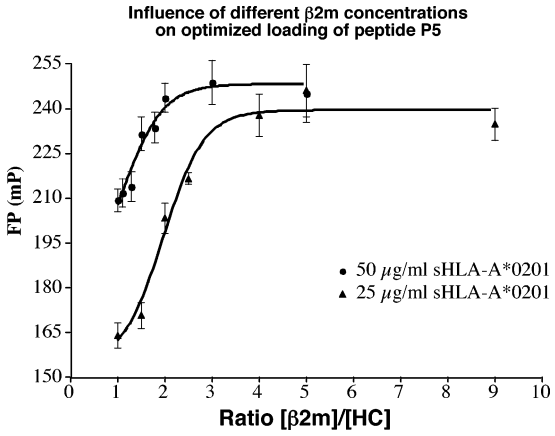

FIGURE 3: Influence of variable β2m concentrations on maximal performance. Fixed amounts of sHLA-A*0201 (50 and 25 μg/mL) and 10 nM pFITC P5 were incubated with varying amounts of β2m expressed as a ratio of [β2m] per [HC] under equilibrium conditions. Results show that peptide loading efficiency was highly increased using excess β2m. An optimal ratio of 3 was determined for both sHLA concentrations meaning that a 2× excess of β2m over sHLA has to be supplemented to achieve maximal polarization levels.

to be more advantageous than activation for 30 or 45 min as a much higher polarization level could be achieved. With longer activation periods, a decline in polarization was observed at temperatures above 50 °C. This decline is due to partial denaturation of the complex by applying too much thermal energy. Most optimal conditions for peptide exchange activation were established by incubating sHLA complexes at 53 °C for 15 min. These parameters were kept constant and were applied for all further experiments. Only a low net increase in polarization was observed at temperatures below 43 °C, and the loading efficiency was comparable to the results obtained through passive loading at room temperature. This observation indicates that our sHLA complexes are very stable and do not efficiently dissociate at temperatures below 43 °C.

To further validate the thermal activation procedure, the structural integrity of sHLA-A*0201 molecules during and after thermal destabilization was monitored using an ELISA-based procedure which uses the conformation-dependent monoclonal antibody W6/32 (*25*). For this stability testing, samples of sHLA-A*0201 were incubated at 58, 53, 47, and 37 °C for various lengths of time. After data transformation to percent relative stability using the nonheated solution (4 °C) of sHLA-A*0201 as reference (100%), data were graphed against time of heat exposure and the half-lives determined (Figure 2). It is shown that the sHLA-A*0201 stability curve rapidly declines at higher temperatures (58 and 53 °C) with half-lives of only 3.7 and 5.7 min, respectively, indicating fast dissociation of the complex into its subunits. After 45 min, the conformational antibody was unable to detect any significant amounts of intact sHLA molecules. This effect was drastically reduced at 47 °C where complete denaturation of most molecules occurred after 3 h and showed a half-life of 51.6 min. Incubation of sHLA-A*0201 at 37 °C demonstrated little effect, and more than 95% of all molecules still maintained their structural integrity.

After heat exposure, selected sHLA preparations were immediately brought to 4 °C and allowed to reassociate for 72 h without supplementation of exogenous peptides or β2m. As seen in Figure 2, a re-formation process of subunits occurred as demonstrated above. Data show that the reassociation of sHLA-A*0201 complexes happens more effectively by using short activation times compared to extensive heat exposure. Incubations over 2 h seem to destroy more than 60% of the molecules, rendering them unusable for FP assays. Interestingly, temperature variance had a lesser effect than the time of heat exposure. Overall, this ELISA approach confirms and validates the thermal activation results obtained by FP experiments. Furthermore, it could also be demonstrated that, under the established conditions for optimal peptide exchange activation (53 °C for 15 min), more than 85% of sHLA-A*0201 molecules are available for the exchange process.

Influence of β2m on Peptide Loading. In order for HLA class I alleles to be able to efficiently bind peptides, recent peptide loading experiments indicated that excess β2m must be present in order to enhance peptide binding (*12*, *27*, *33*−*36*). To further improve the sensitivity of our assay, we tested the influence of variable β2m concentrations on maximal performance. Fixed amounts of sHLA-A*0201 complexes (25/50 μg/mL) were allowed to react with varying amounts of β2m. As shown in Figure 3, addition of higher concentrations of β2m improved binding of the FITC-labeled reference peptide P5 by increasing the dynamic range by 15% for 50 μg/mL and by 30% for 25 μg/mL of sHLA-A*0201, respectively. The response curve for 50 μg/mL compared to the curve generated by 25 μg/mL is higher as more sHLA molecules are available to participate in the binding event. An optimal ratio of three β2m molecules per single HLA heavy chain was determined. Addition of more β2m did not result in a more efficient loading.

As part of the optimization process, another goal was to minimize the contribution of assay components to nonspecific

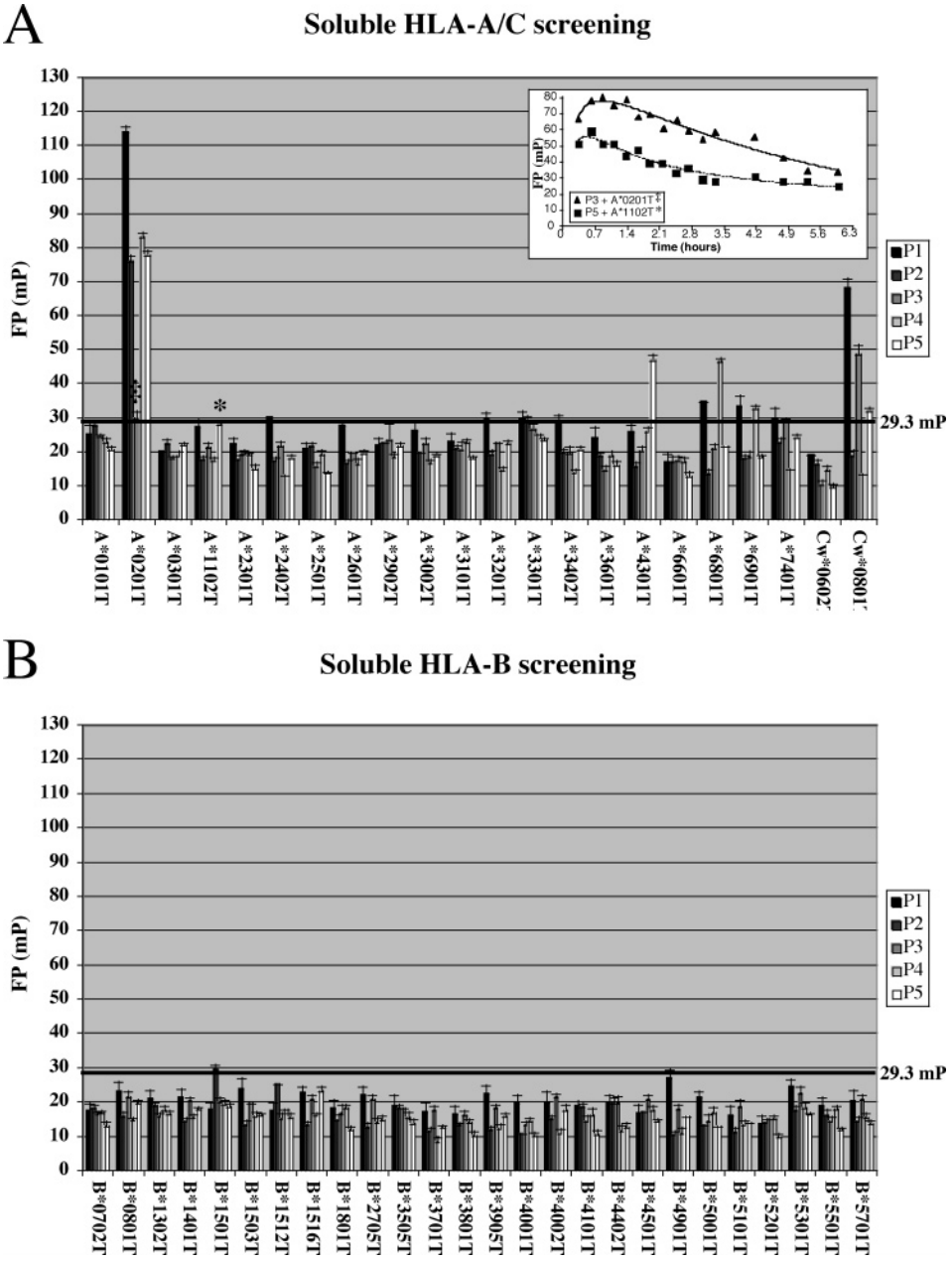

FIGURE 4: Screening of HLA A, B, and C alleles for cross-reactivity evaluation. A panel of 48 distinct sHLA alleles was tested to determine the specificity of our designed peptides to sHLA-A*0201 and to elaborate cross-reactivity to other sHLA allele specificities. FP values are obtained using a more simplified binding protocol without addition of β2m. Each peptide candidate (10 nM) was incubated with 100 μg/mL activated sHLA, and the peptide/HLA interaction was monitored over time. Final equilibrium polarization levels indicating the extent of binding to each allele after incubation with each of our five test peptides are shown. A threshold line of 29.3 mP was introduced, which corresponds to the completely free state (and lowest polarization value) of the labeled ligand. (A) In this graph, 20 HLA-A and 2 HLA-C were tested, of which sHLA-A*0201 and sHLA-Cw*0801 showed the highest reactivity followed by less effective binders such as sHLA-A*4301, sHLA-A*6801, and sHLA-A*6901. The insert visualizes the time course of association for peptide P3 to sHLA-A*0201 (‡) and P5 to sHLA-A*1102 (*). (B) No significant binding was detected to any of the B-alleles tested.

FP. High background counts due to buffer or nonfluorophore components can seriously affect the sensitivity of an assay. By using purified molecules, light scattering through cell membranes and cellular debris was not a problem, and therefore no contribution of the sHLA protein to the final signal could be detected. However, to further improve signal-to-background ratios, we included bovine γ-globulin (BGG) as carrier protein, which drastically decreased baseline fluorescence intensity signals. A concentration of 0.05% BGG was sufficient to prevent nonspecific binding of the FITC-labeled peptides to other assay components and the walls of incubation vessels. In contrast, bovine serum albumin (BSA) completely failed as an alternative because of its ability to bind the fluorescent-labeled peptides, an interaction which greatly increased baseline polarization and reduced assay range (data not shown). Incubation of free FITC molecules with sHLA did not increase FP values, demonstrating that the fluorescent label does not impact the assay. Additional optimization experiments such as testing of various buffer systems did not result in further improvements of the assay protocol and are therefore not presented.

Multiple Specificity Screening. To determine the specificity for sHLA-A*0201 as well as potential cross-reactivity to other sHLA alleles, we screened 48 different sHLA alleles

Table 2: Motif Comparison of Alleles Showing Positive Binding Capacity with A*0201-Specific Peptides

	position												position										
	1	2	3	4	5	6	7	8	9	10	binding		1	2	3	4	5	6	7	8	9	10	binding
A*0201[a]		**L** **M** **V**							**V** **L**			Cw*0801											
P1	F	**L**	P	S	D	*K*	F	P	S	**V**	+	P1	F	**L**	P	S	D	*K*	F	P	S	**V**	+
P4	L	**V**	F	G	*K*	E	V	V	E	**V**	+	P3	N	**L**	S	*K*	L	S	L	D	**V**		+
P5	A	**L**	M	D	K	V	L	*K*	**V**		+	P5	A	**L**	M	D	K	V	L	*K*	**V**		+
P2	S	**L**	Y	N	*K*	V	A	T	**L**		+	P2	S	L	Y	N	*K*	V	A	T	L		−
P3	N	**L**	S	*K*	L	S	L	D	**V**		(+)	P4	L	V	F	G	*K*	E	V	V	E	V	−
A*6801[b]	**D** **E**	**V** **T**							**R** **K**			A*6901[c]		**V** **T** **A**	**I** **F** **L**			**I** **F** **L**			**V** **L**		
P4	L	**V**	F	G	*K*	E	V	V	E	V	+	P4	L	**V**	**F**	G	*K*	E	V	V	E	**V**	+
P1	F	L	P	S	D	*K*	F	P	S	V	+	P1	F	L	P	S	D	*K*	F	P	S	**V**	+
P5	A	L	M	D	K	V	L	*K*	V		−	P5	A	L	M	D	K	V	L	*K*	V		−
P3	N	L	S	*K*	L	S	L	D	V		−	P3	N	L	S	*K*	L	S	L	D	V		−
P2	S	L	Y	N	*K*	V	A	T	L		−	P2	S	L	Y	N	*K*	V	A	T	L		−

[a] See refs *14*, *71*, and *72* for motifs. [b] See ref *73* for motifs. [c] See ref *74* for motifs.

for binding with our A*0201 pFITC constructs (Figure 4). For screening purposes, each peptide candidate (10 nM) was incubated with 100 μg/mL activated sHLA, and the peptide/HLA interaction was monitored over time. As expected, no significant binding was detected to any of the B-alleles tested (Figure 4B), whereas a high binding affinity was observed between sHLA-A*0201 and our pFITC candidates. An exception was pFITC P3 (Figure 4A). This peptide failed to attain a higher polarization level compared to the other candidates. A more detailed investigation showed that, at an early time point of association, this peptide was capable of binding to sHLA-A*0201 (Figure 4A, insert). The significant decrease in association kinetics recorded after 0.8 h seems to imply that the HLA class I molecule is not stabilized following exposure to this peptide, irrespective of whether it attempted to bind. This effect was possibly caused by the integration of the fluorescent tag into the structure as the native peptide is published to have a high affinity to sHLA-A*0201. Thus, modification of the residue at the P4 nonanchor position of pFITC P3 may not affect the specificity and association kinetics of peptide binding but rather seems to affect the stability of the class I peptide interaction.

In addition to a successful interaction with sHLA-A*0201, high to medium binding affinities could also be observed between sHLA-Cw*0801 and peptides P1 and P3. Interestingly, the stability of the sHLA-Cw*0801/P3 interaction was not disrupted as it was for sHLA-A*0201/P3, indicating that positions most suitable for FITC labeling in A*0201 molecules may not be the same as those for Cw*0801. Peptide P5 was also found to bind sHLA-Cw*0801 but to a much lesser extent. However, binding was still significantly over the threshold line (29.3 mP), which corresponds to the determined baseline value where all pFITC conjugates are in a free state. No *G* factor correction was applied for the screening analysis, explaining a threshold of 29.3 mP rather than 27 mP. Comparing the structural characteristics of our designs to each other showed that only those peptides bound to Cw*0801 carrying a leucine (L) at position 2 and valine (V) at the C-terminus. These findings allowed us to propose an initial Cw*0801 binding specificity. Peptides P2 and P4 which did not concur with this rudimentary P2L/P9V motif were not able to bind sHLA-Cw*0801 (Table 2).

Furthermore, binding of peptides P4 and P1 to sHLA-A*6801 and sHLA-A*6901 as well as binding of peptide P5 to sHLA-A*4301 and sHLA-A*1102 could additionally be detected. In case of the interaction of P5 with sHLA-A*1102, the same observation was made as for peptide P3 binding to sHLA-A*0201, showing that this interaction was not stable at equilibrium (Figure 4A, insert). The possibility of having monitored the effect of peptide degradation, however, can be ruled out, as both peptides were capable of binding to other alleles under equal conditions. To support these findings, it has been reported previously that HLA-A*6901 could bind certain A*0201-restricted antigenic peptides (*3*, *37*). Among those peptides is the nonmodified version of the P1 peptide which was also reported to bind A*6802 in addition to A*0201, A*0202, A*0205, and A*0206. However, these alleles were not available for this study.

Generally, screening data showed that four out of the five peptides demonstrated cross-reactivity with at least 2 of the 48 alleles tested, confirming but also extending the cross-reactivity pattern found in other studies. More specifically, P1 and P5 bound to four different sHLA alleles, A*0201, A*6801, A*6901, and Cw*0801 and A*0201, A*4301, Cw*0801, and A*1102, respectively. P4 bound to three different sHLA alleles, A*0201, A*6801, and A*6901, and P3 bound two of the sHLA alleles, A*0201 and Cw*0801, tested. P2 was the only peptide not showing any cross-reactivity and solely interacting with sHLA-A*0201.

*Kinetics of sHLA-A*0201/Peptide Interactions.* We performed a series of experiments to determine the kinetic rate constants to four of our five derivatized peptides. In these experiments, we applied different concentrations of the pFITC ligand by using 50 μg/mL sHLA-A*0201 combined with an excess of β2m at room temperature (20 °C). Under such conditions, peptide replacement kinetics was shown earlier to be independent of the complex or β2m concentrations, therefore reflecting a pseudo-first-order reaction (*38*). After initiation of the reaction, typical curves of peptide binding time courses were recorded following the association of the FITC-labeled peptides to the class I sHLA complex A*0201 (Figure 5). Starting from 27 mP, which corresponds to the free peptide condition, the FP values increased

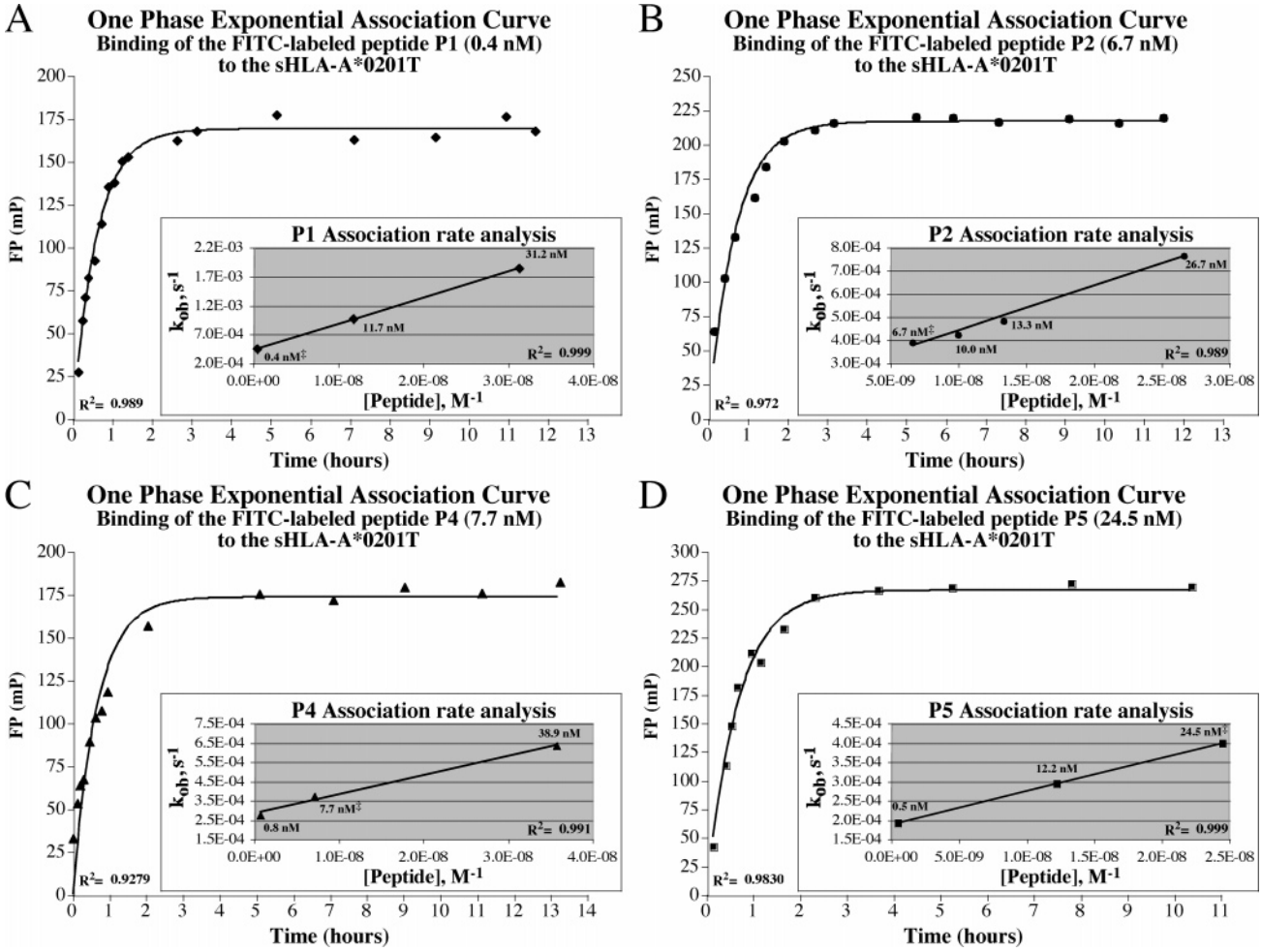

FIGURE 5: Association of FITC-labeled peptides (A) P1, (B) P2, (C) P4, and (D) P5 with sHLA-A*0201. The association profiles for pFITC conjugates with sHLA were recorded at 20 °C at various periods of time in which the FP signals corresponding to the ratio of bound to free pFITC ligand were plotted versus time. Peptide binding to sHLA was rapid and stable and reached a maximum within several hours of incubation depending on the concentration tested. The data were fitted to a monoexponential association model to determine apparent rate constants (k_{ob}). Concentration dependence of pFITC binding kinetics to HLA-A*0201/β2m is shown as inserts. Extrapolation of the plot to zero ligand allowed calculating absolute rate constants for association (k_{on}) and dissociation (k_{off}) as well as equilibrium dissociation constants (K_d^{kin}).

Table 3: Summary of Kinetic and Equilibrium Constants

		A*0201T				
		P1	P2	P4	P5	
association rate constants	k_{on}	4.52	1.94	0.99	8.68	$10^4\ M^{-1}\ s^{-1}$
dissociation rate constants	k_{off}	4.33	2.40	2.84	1.88	$10^{-4}\ s^{-1}$
equilibrium dissociation constants (kinetics)	K_d^{kin}	9.6	12.4	28.5	21.6	10^{-9} M
equilibrium dissociation constants (titration)	K_d^{titr} [a]	10.7	21.1	21.8	17.3	10^{-9} M

[a] K_d^{titr} from Buchli et al., unpublished data.

primarily during the first 3 h of the experiment. After this period, values reached a maximum plateau, where no further increase was monitored, indicating that equilibrium had been reached. The observed (apparent) association rate constant (k_{ob}), which is concentration dependent and expressed in units of inverse time, was obtained by fitting all data points to a monoexponential association model (see Materials and Methods section). As expected, the apparent dissociation rates were slower for low concentrations and faster for higher concentrations. Since the observed association rate constant was increasing linearly with the concentration of peptide, the data obtained allowed the calculation of absolute rate constants for association (k_{on}) and dissociation (k_{off}). Extrapolation of the plot to zero ligand as presented in the Figure 5 inserts, determined the intercept, which equals k_{off}, whereas the slope of the graph corresponds to k_{on}. As listed in Table 3, peptide dissociation rate constants for A*0201 peptide designs tested with sHLA-A*0201 were found to be relatively consistent [$(1.9-4.3) \times 10^{-4}\ s^{-1}$]. This was not surprising considering that all peptides were considered to be high-affinity binders as well as good CTL responders, thus assuming the necessity of low dissociation rates for in vivo immunogenicity (*5*). Equally, the rate constants for association were also found to be very similar [$(1.0-8.7) \times$

10^4 M^{-1} s^{-1}]. Such a close range of association rate constants indicates that all peptides seem to use the same association mechanism.

In addition, the kinetic data presented also allow for the estimation of the equilibrium dissociation constant (K_d^{kin}), which is the ratio of the dissociation and association rate constant ($K_d^{kin} = k_{off}/k_{on}$). All constants determined were in very good agreement with the values obtained in titration experiments under equilibrium conditions (K_d^{titr}) (Buchli et al., unpublished data), further confirming the accuracy of FP-based binding assays (Table 3).

DISCUSSION

To establish a real-time assay to directly evaluate the interaction of HLA class I molecules with synthetic peptides, we have exploited genetically engineered sHLA class I analogues. Compared to native HLA class I glycoproteins, the heavy chains of these recombinant sHLA molecules are truncated following exon 4, just before the transmembrane and cytoplasmic domain. Their reactivity with a panel of monoclonal antibodies specific for HLA class I molecules demonstrated that the soluble trimeric molecules are structurally and functionally intact, maintaining their native conformation (*20*, *21*, *39*). For this study, we combined our sHLA technology with FP, a state-of-the-art detection system for investigating molecular binding interactions. FP binding assays are based on the polarization of a FITC moiety covalently bound to a peptide of interest and differ from other types of binding studies in one important regard: they require no steps to separate free from bound and are therefore fast, simple, and accurate. Data acquisition is relatively simple and rapid, and results are of high quality for detailed analysis of HLA/peptide interactions. In addition, the technique exhibits sufficient sensitivity to replace radioactive detection methods. These advantages make FP an excellent tool for the fast and precise determination of molecular interactions in real time, and FP has already been utilized in a large variety of applications, e.g., protein−DNA interactions (*40*), enzyme−activity assays (*41*), and protein−protein (*24*) and protein−ligand interactions (*42*, *43*). FP observations can be comfortably made at nanomolar concentrations but become more difficult and less reliable at lower concentrations (Buchli et al., unpublished data).

One of the first challenges in the development of such a new procedure was the loading of exogenous synthetic peptides into a fully assembled sHLA complex. The human class I HLA complex is a heterotrimer consisting of a constant light chain of β2m and a class-specific heavy chain to which a specific peptide is bound. In general, it is difficult to demonstrate direct peptide binding to purified class I molecules (*11*, *27*, *44*), presumably because most class I complexes contain endogenous peptides that either dissociate themselves or allow β2m to dissociate. The concept of very slow dissociation of peptides from assembled class I molecules is consistent with our findings of minimal peptide binding in solution at room temperature (Figure 1A) as well as under physiological conditions (Figure 1B). Therefore, if a complex is held together by many bonds, then a fluctuation in which enough bonds are simultaneously ruptured to allow the complex to fall apart will be very rare. To overcome such barriers, we designed a peptide exchange procedure in which the original endogenous peptide is first released from the HLA binding groove by incubating sHLA samples for 15 min at 53 °C. Peptide interactions have been described as a function of ionic forces, hydrophobic interactions, and hydrogen bond-type interactions. Thermal treatment can weaken these bonds and lead to enhanced peptide exchange. Temperature dependence of peptide binding to the heavy chain has been demonstrated earlier (*45*) and is consistent with previous thermal stability studies showing that the degree of heavy chain unfolding is less than 10% at 53 °C (*31*), and an HLA/peptide complex can exhibit proper refolding even after being heated to 80 °C (*46*). However, as demonstrated in this study, the extent of energy employed has to be carefully controlled to ensure that the majority of molecules are capable of restoring proper conformation after the reinstatement of original conditions. Too much denaturation caused irreversible damage as seen by prolonged incubations at higher temperatures (Figure 1B and Figure 2).

Comparison of experiments done with equimolar concentrations of the heavy chain and β2m with those done using an excess of β2m showed that excess β2m concentrations shift the polarization signal toward complex formation. A number of groups have previously reported enhanced peptide binding by cell surface or purified class I molecules in the presence of excess free β2m (*12*, *33*−*35*, *47*). Since assembly of class I heavy chains with β2m is accompanied by a characteristic change in conformation of the α1 and α2 domains (*48*−*50*), it was suggested that β2m in excess can stabilize folding of the α1 and α2 domains of the heavy chain without addition of peptides (*47*, *50*−*53*) and consequently enhance peptide binding. In addition, as shown earlier (*27*, *38*, *54*−*56*), peptide binding seems not to require β2m dissociation from the HLA heavy chain. Considering the presence of excess β2m combined with the fact that the rate constant of heavy chain association with β2m is much faster than that with peptides (*45*), the presence of a majority of heterodimers is very reasonable under such activated conditions. Therefore, heterodimers consisting of heavy chain and β2m may be sufficiently stable to allow reassembly of the A*0201 protein complex with labeled peptides without complete separation into single subunits. In such a reassociation reaction, the formation of a new complex seems to require only a collision between the heterodimer and the labeled peptide. The subsequent formation of just enough weak bonds to keep the complex together as more and more electrostatic interactions, hydrogen bonds, and hydrophobic interactions occur may gradually increase the stability of the complex.

The physiological relevance of the k_{on} of peptides for preformed class I molecules is still unclear, because peptides can bind class I in vitro either during or after assembly of the heavy chain/β2m complex (*6*, *47*) and may interact with free heavy chain prior to association with β2m (*57*). In contrast, k_{off} seems to have more physiological relevance. Our test results show that the dissociation rate constants for the interaction of sHLA-A*0201 with the derivatized peptides P1, P2, P4, and P5 were in a low range of 1.9×10^{-4} to 4.3×10^{-4} s^{-1}, supporting a relationship between k_{off} rates and immunogenicity as native peptides for P1, P2, and P4 are known to be highly immunogenic. For peptide association, it is generally agreed that the interaction between peptide

ligands and HLA molecules is a complex process involving multiple steps between the initial interaction and the fully complex state (*58*). Therefore, any process which would alter one or more of these binding steps would result in a change in the apparent association rate constant. Testing of several peptides using sHLA-A*0201 did not reveal major differences in association rate constants [$(1.0-8.7) \times 10^4$ M^{-1} s^{-1}] ,which would indicate that all peptides use the same association mechanism. Several studies have reported good correlation between binding kinetics and the degree of T cell activation, but an absolute correlation between binding kinetics and biological effect has still not been experimentally observed (*59*, *60*).

Overall, the obtained kinetic rate values of the engineered sHLA molecules are remarkably close to those previously obtained for native molecules in human (*15*, *38*, *58*, *61*−*65*) as well as in murine systems (*62*, *65*−*69*), indicating that these soluble heterodimers bind in the same manner as the original molecules expressed on cells. In general, the reassembly pathway for sHLA/peptide complexes under our assay conditions seems to be best described by the binding of peptide to preformed heavy chain/β2m heterodimer as suggested in the K2 step described by Matsumura (*58*). Finally, it is evident that labeling at positions P5 and P8 for 9-mers and at positions P5 and P6 for 10-mers, respectively, does not interfere significantly with ligand binding. These results are supported by a variety of publications demonstrating the successful attachment of a label at positions 5, 6, 7, and 8 in nonamer (*15*, *36*, *44*, *65*) and positions 5, 6, 7, 8, and 9 in decamer peptides (*5*, *15*, *65*, *67*, *70*).

In conclusion, this study clearly demonstrates the exceptional value of real-time FP-based analysis to quantitate HLA/peptide interactions. The assay offers great sensitivity and specificity, does not depend on radioactivity, is simple to conduct, and permits a direct view of the HLA/peptide dynamics. With the utilization of purified, high-quality sHLA molecules, which are available in large quantities, this assay exceeds the current expectations of a quantitative peptide/HLA binding assay and will open new horizons in the field of high-throughput epitope discovery.

ACKNOWLEDGMENT

We thank Pierre Chrétien for informative scientific discussions and critical review. Mrs. Beatrice Buchli is acknowledged for assistance with the preparation of the manuscript.

REFERENCES

1. Schumacher, T. N., Heemels, M. T., Neefjes, J. J., Kast, W. M., Melief, C. J., and Ploegh, H. L. (1990) Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro, *Cell 62*, 563−567.
2. Hosken, N. A., and Bevan, M. J. (1990) Defective presentation of endogenous antigen by a cell line expressing class I molecules, *Science 248*, 367−370.
3. Storkus, W. J., Zeh, H. J., III, Salter, R. D., and Lotze, M. T. (1993) Identification of T-cell epitopes: rapid isolation of class I-presented peptides from viable cells by mild acid elution, *J. Immunother. 14*, 94−103.
4. van der Burg, S. H., Klein, M. R., van de Velde, C. J., Kast, W. M., Miedema, F., and Melief, C. J. (1995) Induction of a primary human cytotoxic T-lymphocyte response against a novel conserved epitope in a functional sequence of HIV-1 reverse transcriptase, *AIDS 9*, 121−127.
5. van der Burg, S. H., Visseren, M. J., Brandt, R. M., Kast, W. M., and Melief, C. J. (1996) Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability, *J. Immunol. 156*, 3308−3314.
6. Townsend, A., Ohlen, C., Bastin, J., Ljunggren, H. G., Foster, L., and Karre, K. (1989) Association of class I major histocompatibility heavy and light chains induced by viral peptides, *Nature 340*, 443−448.
7. Huczko, E. L., Bodnar, W. M., Benjamin, D., Sakaguchi, K., Zhu, N. Z., Shabanowitz, J., Henderson, R. A., Appella, E., Hunt, D. F., and Engelhard, V. H. (1993) Characteristics of endogenous peptides eluted from the class I MHC molecule HLA-B7 determined by mass spectrometry and computer modeling, *J. Immunol. 151*, 2572−2587.
8. Zeh, H. J., III, Leder, G. H., Lotze, M. T., Salter, R. D., Tector, M., Stuber, G., Modrow, S., and Storkus, W. J. (1994) Flow-cytometric determination of peptide-class I complex formation. Identification of p53 peptides that bind to HLA-A2, *Hum. Immunol. 39*, 79−86.
9. Boisgerault, F., Tieng, V., Stolzenberg, M. C., Dulphy, N., Khalil, I., Tamouza, R., Charron, D., and Toubert, A. (1996) Differences in endogenous peptides presented by HLA-B*2705 and B*2703 allelic variants. Implications for susceptibility to spondylarthropathies, *J. Clin. Invest. 98*, 2764−2770.
10. Buus, S., Sette, A., Colon, S. M., Jenis, D. M., and Grey, H. M. (1986) Isolation and characterization of antigen-Ia complexes involved in T cell recognition, *Cell 47*, 1071−1077.
11. Chen, B. P., and Parham, P. (1989) Direct binding of influenza peptides to class I HLA molecules, *Nature 337*, 743−745.
12. Olsen, A. C., Pedersen, L. O., Hansen, A. S., Nissen, M. H., Olsen, M., Hansen, P. R., Holm, A., and Buus, S. (1994) A quantitative assay to measure the interaction between immunogenic peptides and purified class I major histocompatibility complex molecules, *Eur. J. Immunol. 24*, 385−392.
13. Khilko, S. N., Corr, M., Boyd, L. F., Lees, A., Inman, J. K., and Margulies, D. H. (1993) Direct detection of major histocompatibility complex class I binding to antigenic peptides using surface plasmon resonance. Peptide immobilization and characterization of binding specificity, *J. Biol. Chem. 268*, 15425−15434.
14. Ruppert, J., Sidney, J., Celis, E., Kubo, R. T., Grey, H. M., and Sette, A. (1993) Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules, *Cell 74*, 929−937.
15. Sette, A., Sidney, J., del Guercio, M. F., Southwood, S., Ruppert, J., Dahlberg, C., Grey, H. M., and Kubo, R. T. (1994) Peptide binding to the most frequent HLA-A class I alleles measured by quantitative molecular binding assays, *Mol. Immunol. 31*, 813−822.
16. Buus, S., Stryhn, A., Winther, K., Kirkby, N., and Pedersen, L. O. (1995) Receptor-ligand interactions measured by an improved spun column chromatography technique. A high efficiency and high throughput size separation method, *Biochim. Biophys. Acta 1243*, 453−460.
17. Fruci, D., Rovero, P., Butler, R. H., Sorrentino, R., Tosi, R., and Tanigaki, N. (1993) HLA class I binding of synthetic nonamer peptides carrying major anchor residue motifs of HLA-B27 (B*2705)-binding peptides, *Immunogenetics 38*, 41−46.
18. Gnjatic, S., Bressac-de Paillerets, B., Guillet, J. G., and Choppin, J. (1995) Mapping and ranking of potential cytotoxic T epitopes in the p53 protein: effect of mutations and polymorphism on peptide binding to purified and refolded HLA molecules, *Eur. J. Immunol. 25*, 1638−1642.
19. Silver, M. L., Parker, K. C., and Wiley, D. C. (1991) Reconstitution by MHC-restricted peptides of HLA-A2 heavy chain with beta 2-microglobulin, in vitro, *Nature 350*, 619−622.
20. Prilliman, K., Lindsey, M., Zuo, Y., Jackson, K. W., Zhang, Y., and Hildebrand, W. (1997) Large-scale production of class I bound peptides: assigning a signature to HLA-B*1501, *Immunogenetics 45*, 379−385.
21. Prilliman, K. R., Lindsey, M., Jackson, K. W., Cole, J., Bonner, R., and Hildebrand, W. H. (1998) Complexity among constituents of the HLA-B*1501 peptide motif, *Immunogenetics 48*, 89−97.
22. Perrin, F. (1926) Polarization of light fluorescence, average life of molecules in the excited state, *J. Phys. Radium 7*, 390−401.
23. Checovich, W. J., Bolger, R. E., and Burke, T. (1995) Fluorescence polarization—a new tool for cell and molecular biology, *Nature 375*, 254−256.
24. Jameson, D. M., and Seifried, S. E. (1999) Quantification of protein−protein interactions using fluorescence polarization, *Methods 19*, 222−233.

25. Barnstable, C. J., Bodmer, W. F., Brown, G., Galfre, G., Milstein, C., Williams, A. F., and Ziegler, A. (1978) Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis, *Cell 14*, 9−20.
26. Herron, J. N., and Voss, E. W., Jr. (1981) Characterization of fluorescent 2-dimethylaminonaphthalene-5-sulfonyl-immunoglobulin G conjugates for application in fluorescence polarization studies, *J. Biochem. Biophys. Methods 5*, 1−17.
27. Parker, K. C., DiBrino, M., Hull, L., and Coligan, J. E. (1992) The beta 2-microglobulin dissociation rate is an accurate measure of the stability of MHC class I heterotrimers and depends on which peptide is bound, *J. Immunol. 149*, 1896−1904.
28. Tanigaki, N., Fruci, D., Chersi, A., and Butler, R. H. (1993) Unfolded HLA class I alpha chains and their use in an assay of HLA class-I-peptide binding, *Hum. Immunol. 36*, 119−127.
29. Stryhn, A., Pedersen, L. O., Romme, T., Olsen, A. C., Nissen, M. H., Thorpe, C. J., and Buus, S. (1996) pH dependence of MHC class I-restricted peptide presentation, *J. Immunol. 156*, 4191−4197.
30. Jensen, P. E., Moore, J. C., and Lukacher, A. E. (1998) A europium fluoroimmunoassay for measuring peptide binding to MHC class I molecules, *J. Immunol. Methods 215*, 71−80.
31. Reinelt, S., Dedier, S., Asuni, G., Folkers, G., and Rognan, D. (2001) Mutation of Cys-67 alters the thermodynamic stability of the human leukocyte antigen HLA0-B*2705, *J. Biol. Chem. 276*, 18472−18477.
32. Benjamin, R. J., Madrigal, J. A., and Parham, P. (1991) Peptide binding to empty HLA-B27 molecules of viable human cells, *Nature 351*, 74−77.
33. Vitiello, A., Potter, T. A., and Sherman, L. A. (1990) The role of beta 2-microglobulin in peptide binding by class I molecules, *Science 250*, 1423−1426.
34. Kozlowski, S., Takeshita, T., Boehncke, W. H., Takahashi, H., Boyd, L. F., Germain, R. N., Berzofsky, J. A., and Margulies, D. H. (1991) Excess beta 2 microglobulin promoting functional peptide association with purified soluble class I MHC molecules, *Nature 349*, 74−77.
35. Rock, K. L., Gamble, S., Rothstein, L., Gramm, C., and Benacerraf, B. (1991) Dissociation of beta 2-microglobulin leads to the accumulation of a substantial pool of inactive class I MHC heavy chains on the cell surface, *Cell 65*, 611−620.
36. Gakamsky, D. M., Davis, D. M., Strominger, J. L., and Pecht, I. (2000) Assembly and dissociation of human leukocyte antigen (HLA)-A2 studied by real-time fluorescence resonance energy transfer, *Biochemistry 39*, 11163−11169.
37. del Guercio, M. F., Sidney, J., Hermanson, G., Perez, C., Grey, H. M., Kubo, R. T., and Sette, A. (1995) Binding of a peptide antigen to multiple HLA alleles allows definition of an A2-like supertype, *J. Immunol. 154*, 685−693.
38. Gakamsky, D. M., Bjorkman, P. J., and Pecht, I. (1996) Peptide interaction with a class I major histocompatibility complex-encoded molecule: allosteric control of the ternary complex stability, *Biochemistry 35*, 14841−14848.
39. Prilliman, K. R., Lindsey, M., Wang, J., Jackson, K. W., and Hildebrand, W. H. (1999) Peptide motif of the class I molecule HLA-B*1503, *Immunogenetics 49*, 144−146.
40. Aucouturier, P., Preud'homme, J. L., and Lubochinsky, B. (1983) Fluorescence polarization immunoassay of estradiol, *Diagn. Immunol. 1*, 310−314.
41. Levine, L. M., Michener, M. L., Toth, M. V., and Holwerda, B. C. (1997) Measurement of specific protease activity utilizing fluorescence polarization, *Anal. Biochem. 247*, 83−88.
42. Dedier, S., Reinelt, S., Rion, S., Folkers, G., and Rognan, D. (2001) Use of fluorescence polarization to monitor MHC-peptide interactions in solution, *J. Immunol. Methods 255*, 57−66.
43. Lynch, B. A., Loiacono, K. A., Tiong, C. L., Adams, S. E., and MacNeil, I. A. (1997) A fluorescence polarization based Src-SH2 binding assay, *Anal. Biochem. 247*, 77−82.
44. Tsomides, T. J., Walker, B. D., and Eisen, H. N. (1991) An optimal viral peptide recognized by CD8+ T cells binds very tightly to the restricting class I major histocompatibility complex protein on intact cells but not to the purified class I protein, *Proc. Natl. Acad. Sci. U.S.A. 88*, 11276−11280.
45. Gakamsky, D. M., Boyd, L. F., Margulies, D. H., Davis, D. M., Strominger, J. L., and Pecht, I. (1999) An allosteric mechanism controls antigen presentation by the H-2K(b) complex, *Biochemistry 38*, 12165−12173.
46. Fahnestock, M. L., Tamir, I., Narhi, L., and Bjorkman, P. J. (1992) Thermal stability comparison of purified empty and peptide-filled forms of a class I MHC molecule, *Science 258*, 1658−1662.
47. Townsend, A., Elliott, T., Cerundolo, V., Foster, L., Barber, B., and Tse, A. (1990) Assembly of MHC class I molecules analyzed in vitro, *Cell 62*, 285−295.
48. Williams, D. B., Barber, B. H., Flavell, R. A., and Allen, H. (1989) Role of beta 2-microglobulin in the intracellular transport and surface expression of murine class I histocompatibility molecules, *J. Immunol. 142*, 2796−2806.
49. Allen, H., Fraser, J., Flyer, D., Calvin, S., and Flavell, R. (1986) Beta 2-microglobulin is not required for cell surface expression of the murine class I histocompatibility antigen H-2Db or of a truncated H-2Db, *Proc. Natl. Acad. Sci. U.S.A. 83*, 7447−7451.
50. Myers, N. B., Lie, W. R., Nett, M., Rubocki, R. J., and Hansen, T. H. (1989) The conformation of Ld induced by beta 2-microglobulin is fixed during de novo synthesis and irreversible by exchange or dissociation, *J. Immunol. 142*, 2751−2758.
51. Smith, M. H., and Barber, B. H. (1990) The conformational flexibility of class I H-2 molecules as revealed by anti-peptide antibodies specific for intracytoplasmic determinants: differential reactivity of beta 2-microglobulin "bound" and "free" H-2Kb heavy chains, *Mol. Immunol. 27*, 169−180.
52. Morgan, C. L., Ruprai, A. K., Solache, A., Lowdell, M., Price, C. P., Cohen, S. B., Parham, P., Madrigal, J. A., and Newman, D. J. (1998) The influence of exogenous peptide on beta2-microglobulin exchange in the HLA complex: analysis in real-time, *Immunogenetics 48*, 98−107.
53. Allen, H., Wraith, D., Pala, P., Askonas, B., and Flavell, R. A. (1984) Domain interactions of H-2 class I antigens alter cytotoxic T-cell recognition sites, *Nature 309*, 279−281.
54. Smith, J. D., Lie, W. R., Gorka, J., Myers, N. B., and Hansen, T. H. (1992) Extensive peptide ligand exchange by surface class I major histocompatibility complex molecules independent of exogenous beta 2-microglobulin, *Proc. Natl. Acad. Sci. U.S.A. 89*, 7767−7771.
55. Cook, J. R., Myers, N. B., and Hansen, T. H. (1996) The mechanisms of peptide exchange and beta 2-microglobulin exchange on cell surface Ld and Kb molecules are noncooperative, *J. Immunol. 157*, 2256−2261.
56. Horig, H., Papadopoulos, N. J., Vegh, Z., Palmieri, E., Angeletti, R. H., and Nathenson, S. G. (1997) An in vitro study of the dynamic features of the major histocompatibility complex class I complex relevant to its role as a versatile peptide-receptive molecule, *Proc. Natl. Acad. Sci. U.S.A. 94*, 13826−13831.
57. Elliott, T. (1991) How do peptides associate with MHC class I molecules?, *Immunol. Today 12*, 386−388.
58. Matsumura, M., Saito, Y., Jackson, M. R., Song, E. S., and Peterson, P. A. (1992) In vitro peptide binding to soluble empty class I major histocompatibility complex molecules isolated from transfected *Drosophila melanogaster* cells, *J. Biol. Chem. 267*, 23589−23595.
59. Baker, B. M., Gagnon, S. J., Biddison, W. E., and Wiley, D. C. (2000) Conversion of a T cell antagonist into an agonist by repairing a defect in the TCR/peptide/MHC interface: implications for TCR signaling, *Immunity 13*, 475−484.
60. Kersh, G. J., Kersh, E. N., Fremont, D. H., and Allen, P. M. (1998) High- and low-potency ligands with similar affinities for the TCR: the importance of kinetics in TCR signaling, *Immunity 9*, 817−826.
61. Binz, A. K., Rodriguez, R. C., Biddison, W. E., and Baker, B. M. (2003) Thermodynamic and kinetic analysis of a peptide-class I MHC interaction highlights the noncovalent nature and conformational dynamics of the class I heterotrimer, *Biochemistry 42*, 4954−4961.
62. Ojcius, D. M., Godeau, F., Abastado, J. P., Casanova, J. L., and Kourilsky, P. (1993) Real-time measurement of antigenic peptide binding to empty and preloaded single-chain major histocompatibility complex class I molecules, *Eur. J. Immunol. 23*, 1118−1124.
63. Springer, S., Doring, K., Skipper, J. C., Townsend, A. R., and Cerundolo, V. (1998) Fast association rates suggest a conformational change in the MHC class I molecule H-2Db upon peptide binding, *Biochemistry 37*, 3001−3012.
64. Burshtyn, D. N., and Barber, B. H. (1993) Dynamics of peptide binding to purified antibody-bound H-2Db and H-2Db beta 2m complexes, *J. Immunol. 151*, 3082−3093.
65. Khilko, S. N., Jelonek, M. T., Corr, M., Boyd, L. F., Bothwell, A. L., and Margulies, D. H. (1995) Measuring interactions of MHC

class I molecules using surface plasmon resonance, *J. Immunol. Methods 183*, 77−94.
66. Cerundolo, V., Elliott, T., Elvin, J., Bastin, J., Rammensee, H. G., and Townsend, A. (1991) The binding affinity and dissociation rates of peptides for class I major histocompatibility complex molecules, *Eur. J. Immunol. 21*, 2069−2075.
67. Chen, W., Khilko, S., Fecondo, J., Margulies, D. H., and McCluskey, J. (1994) Determinant selection of major histocompatibility complex class I-restricted antigenic peptides is explained by class I-peptide affinity and is strongly influenced by nondominant anchor residues, *J. Exp. Med. 180*, 1471−1483.
68. Luescher, I. F., Loez, J. A., Malissen, B., and Cerottini, J. C. (1992) Interaction of antigenic peptides with MHC class I molecules on living cells studied by photoaffinity labeling, *J. Immunol. 148*, 1003−1011.
69. Neefjes, J. J., Dierx, J., and Ploegh, H. L. (1993) The effect of anchor residue modifications on the stability of major histocompatibility complex class I-peptide interactions, *Eur. J. Immunol. 23*, 840−845.
70. Kessler, J. H., Mommaas, B., Mutis, T., Huijbers, I., Vissers, D., Benckhuijsen, W. E., Schreuder, G. M., Offringa, R., Goulmy, E., Melief, C. J., van der Burg, S. H., and Drijfhout, J. W. (2003) Competition-based cellular peptide binding assays for 13 prevalent HLA class I alleles using fluorescein-labeled synthetic peptides, *Hum. Immunol. 64*, 245−255.
71. Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H. G. (1991) Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules, *Nature 351*, 290−296.
72. Kubo, R. T., Sette, A., Grey, H. M., Appella, E., Sakaguchi, K., Zhu, N. Z., Arnott, D., Sherman, N., Shabanowitz, J., Michel, H., et al. (1994) Definition of specific peptide motifs for four major HLA-A alleles, *J. Immunol. 152*, 3913−3924.
73. Guo, H. C., Jardetzky, T. S., Garrett, T. P., Lane, W. S., Strominger, J. L., and Wiley, D. C. (1992) Different length peptides bind to HLA-Aw68 similarly at their ends but bulge out in the middle, *Nature 360*, 364−366.
74. Barouch, D., Friede, T., Stevanovic, S., Tussey, L., Smith, K., Rowland-Jones, S., Braud, V., McMichael, A., and Rammensee, H. G. (1995) HLA-A2 subtypes are functionally distinct in peptide binding and presentation, *J. Exp. Med. 182*, 1847−1856.

BI048580Q