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

Kessler, J.

CHAPTER 2

CHAPTER 5

Published in
J. Exp. Med. 193:73-88, 2001

Efficient Identification of Novel HLA-A*0201–presented Cytotoxic T Lymphocyte Epitopes in the Widely Expressed Tumor Antigen PRAME by Proteasome-mediated Digestion Analysis

By Jan H. Kessler,* Nico J. Beekman,* Sandra A. Bres-Vloemans,* Pauline Verdijk,* Peter A. van Veelen,* Antoinette M. Kloosterman-Joosten,* Debby C.J. Vissers,* George J.A. ten Bosch,* Michel G.D. Kester,* Alice Sijts,‡ Jan Wouter Drijfhout,* Ferry Ossendorp,* Rienk Offringa,* and Cornelis J.M. Melief*

*From the *Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, 2300 RC Leiden, The Netherlands; and the ‡Institute of Biochemistry, Charité, Humboldt University, D-10117 Berlin, Germany*

Abstract

We report the efficient identification of four human histocompatibility leukocyte antigen (HLA)-A*0201–presented cytotoxic T lymphocyte (CTL) epitopes in the tumor-associated antigen PRAME using an improved "reverse immunology" strategy. Next to motif-based HLA-A*0201 binding prediction and actual binding and stability assays, analysis of in vitro proteasome-mediated digestions of polypeptides encompassing candidate epitopes was incorporated in the epitope prediction procedure. Proteasome cleavage pattern analysis, in particular determination of correct COOH-terminal cleavage of the putative epitope, allows a far more accurate and selective prediction of CTL epitopes. Only 4 of 19 high affinity HLA-A*0201 binding peptides (21%) were found to be efficiently generated by the proteasome in vitro. This approach avoids laborious CTL response inductions against high affinity binding peptides that are not processed and limits the number of peptides to be assayed for binding. CTL clones induced against the four identified epitopes (VLDGLDVLL, $PRA^{100–108}$; SLYSFPEPEA, $PRA^{142–151}$; ALYVDSLFFL, $PRA^{300–309}$; and SLLQHLIGL, $PRA^{425–433}$) lysed melanoma, renal cell carcinoma, lung carcinoma, and mammary carcinoma cell lines expressing PRAME and HLA-A*0201. This indicates that these epitopes are expressed on cancer cells of diverse histologic origin, making them attractive targets for immunotherapy of cancer.

Key words: antigen presentation • antigen processing • cytotoxic T lymphocyte induction • human histocompatibility leukocyte antigen class I binding • tumor immunotherapy

Introduction

T cell–based immunotherapy of cancer has been successful in numerous mouse tumor model systems (1) and so far efficacious in a limited number of clinical conditions (2–6). Specific T cell–mediated immunotherapy requires the identification of tumor-specific antigens carrying T cell epitopes presented in the context of HLA class I and/or HLA class II molecules (for reviews, see references 1 and 7–9). The strategy, pioneered by Boon and coworkers, of screening melanoma cDNA libraries with CTLs derived from tumor infiltrating lymphocytes, has been successful in the identification of CTL epitopes in unknown tumor-associated proteins. The MAGE, BAGE, and GAGE families of tumor-associated testis-specific antigens (10–13), as well as the melanocyte differentiation antigens overexpressed in tumors like tyrosinase, Melan-A/MART-1, and gp100 (14–17) have been identified in this manner. This strategy requires the availability of CTL clones from mixed leukocyte tumor cultures of cancer patients. Consequently, any CTL epitope that is not successful in activating CTLs

Address correspondence to J. Kessler, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Building 1: E3-Q, P.O. Box 9600, 2300 RC Leiden, The Netherlands. Phone: 31-71-526-1671; Fax: 31-71-521-6751; E-mail: kesslerj@worldonline.nl

that can be expanded in vitro (e.g., subdominant epitopes) will be missed. For the systematic detection of CTL epitopes presented in a broad range of HLA class I molecules, there is a great need for an efficient strategy. The strategy of predicting potential CTL epitopes in already identified tumor-associated proteins followed by in vitro sensitization of CTLs against these putative epitopes (also designated "reverse immunology") has the advantage that it does not utilize patient-derived T cells as a primary screen and therefore allows a more systematic search for new CTL epitopes. Reverse immunology has been used to identify new CTL epitopes in MAGE-1 (18), MAGE-2 (19), MAGE-3 (20–23), TRP2 (24), gp100 (25), and HER-2/neu (19). However, so far this strategy has been rather inefficient, mainly because CTLs raised against putative epitopes were often unable to recognize tumor cells expressing the source protein (26–30). Explanations for these failures are both an insufficient affinity of the induced CTLs for their MHC–peptide complex but more often the lack of processing of the presumed epitope (9). Recently, Chaux et al. successfully applied an alternative strategy of in vitro CTL inductions against dendritic cells (DCs)[1] transduced with MAGE-1, abandoning the strategy of epitope prediction and allowing processing of the relevant epitopes to take place by the APC (31).

In this study, we chose to improve the epitope prediction strategy by verifying the proteasome-mediated generation of peptides in order to identify HLA-A*0201–presented CTL epitopes in the so-called PRAME protein. The main intracellular mechanisms that define the exact amino acid (aa) sequence of a CTL epitope include enzymatic breakdown of the protein by the proteasome, transporter-associated with antigen processing (TAP)-mediated translocation into the endoplasmic reticulum (ER) and binding of the peptide with sufficient affinity in the groove of an MHC class I molecule (for reviews, see references 32 and 33). The COOH terminus of CTL epitopes requires exact cleavage by the proteasome (34–37), whereas NH_2-terminal extensions of the epitope can apparently be trimmed by putative aminopeptidase activity mainly in the ER (36, 38–41) or in the cytosol (34, 42). In vitro proteasome-mediated digestions are known to reliably yield MHC class I ligands from viral and model protein-derived polypeptides (43–47). Therefore, after identification of HLA-A*0201 binding peptides, we now incorporated in vitro proteasome-mediated digestions of 27-mer polypeptides encompassing high affinity binding peptides in the epitope prediction procedure. Digestion pattern analysis permitted assessment of efficient COOH-terminal generation of putative epitopes and, in addition, enabled evaluation of possible premature destruction by major cleavage sites within the epitope, as observed by us in a variant viral sequence (47).

[1]*Abbreviations used in this paper:* aa, amino acid; B-LCL, B lymphoblastoid cell line; DC, dendritic cell; ER, endoplasmic reticulum; FI, fluorescence index; FL, fluorescein; HBV, hepatitis B virus; MS, mass spectrometry; RT, reverse transcription; TAP, transporter-associated with antigen processing.

The tumor-associated PRAME protein (48) is a particularly attractive target of T cell–based immunotherapy of cancer because of its expression in a wide variety of tumors, including melanoma (95% of patients), renal cell cancer (41%), lung cancer (50%), mammary cancer (27%), acute leukemias (30%), and multiple myeloma (52%; references 49–51), and because of its absence from normal tissues, except testis, and its very low levels in endometrium, ovaries and adrenals. We focussed on HLA-A*0201 as a restriction element because of its high prevalence (45.8%) among the Caucasian population (52). Via the improved multistep epitope prediction procedure, we report here the identification of four naturally processed HLA-A*0201–presented CTL epitopes in PRAME that are recognized by CTLs on cell lines derived from tumors of various histologic origins. This study underscores the importance of incorporating processing criteria for accurate identification of CTL epitopes.

Materials and Methods

Cell Lines and Culture Conditions. The EBV-transformed B lymphoblastoid cell line (B-LCL) JY was cultured in complete culture medium consisting of IMDM (BioWhittaker) supplemented with 8% FCS (Greiner), 100 IU/ml penicillin, and 2 mM L-glutamine. The processing-defective T2 cell line was a gift from Dr. P. Cresswell (Yale University, New Haven, CT). Melanoma cell lines (Mel603, M453, and FM3), renal cell carcinoma cell lines (MZ1851, MZ1774, and MZ1257), and mammary carcinoma cell line MCF7 were provided by Dr. P. Schrier (Leiden University Medical Center). Mammary carcinoma cell line ZR-75-1 was obtained from the American Type Culture Collection. Lung carcinoma cell lines GLC02 and GLC36 were provided by Dr. L. de Leij (University of Groningen, Groningen, The Netherlands). The CD40 ligand–transfected mouse L cell line (53) that was used for generation of activated B cells was donated by Dr. C. van Kooten (Leiden University Medical Center). PRAME cDNA was provided by Dr. P. Coulie (Ludwig Institute for Cancer Research, Brussels, Belgium). The PRAME-encoding insert was cloned into vector pDR2 (Invitrogen), conferring hygromycine resistance. The renal cell carcinoma cell line MZ1851 was transfected with pDR2-PRAME using Fugene (Boehringer) as transfection reagent. After 48 h, hygromycine (100 μg/ml) was added to select transfected cells. Hygromycine-resistant cells were tested by reverse transcription (RT)-PCR for PRAME expression.

Peptides. Peptides were synthesized by solid phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422) using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry. Short peptides for CTL inductions were dissolved in 20 μl DMSO, diluted in 0.9% NaCl to a peptide concentration of 1 mg/ml, and stored at −20°C before usage. The fluorescein (FL)-labeled reference peptide as used in the competition-based HLA-A*0201 binding assay was synthesized, labeled, and characterized as described earlier (54). The sequence of the FL-labeled peptide was FLPSDYFPSV (hepatitis B virus [HBV] nucleocapsid 18–27; reference 55) wherein we substituted the tyrosine with a cysteine to tag an FL group to the peptide: FLPSDC(FL)FPSV (54). The 27-mer polypeptides used for in vitro proteasome digestion were synthesized as described above, purified by reversed phase-HPLC in an acetonitrile–water gradient and lyophilized from acetonitrile–water overnight. Purity was confirmed by mass spectrometry.

*Cellular Competition–based HLA-A*0201 Peptide Binding Assay.* The affinity of peptides for HLA-A*0201 was analyzed using the homozygous HLA-A*0201$^+$ B-LCL JY as described previously (54), with minor adaptations. In brief, naturally bound peptides were stripped from the HLA-A*0201 molecules by exposing the JY cells for 90 s to ice-cold citric acid buffer with pH 3.1 (1:1 mixture of 0.263 M citric acid and 0.123 M Na_2HPO_4). Cells were immediately buffered with ice-cold IMDM containing 2% FCS, washed twice in the same medium, and resuspended in 2% FCS/IMDM containing 2 μg/ml human β_2-microglublin (Sigma-Aldrich). Subsequently, the stripped JY cells were plated at 4×10^4/well in a 96-well V-bottomed plate together with 150 nM of a known high affinity HLA-A2*0201 binding FL-labeled reference peptide (55) and titrated concentrations of competitor test peptide. After incubation for 24 h at 4°C, cells were washed three times in PBS containing 1% BSA, fixed with 0.5% paraformaldehyde, and analyzed on a FACScan™ flow cytometer (Becton Dickinson). The percentage inhibition of FL-labeled reference peptide binding was calculated using the following formula: $[1 - (MF_{\text{reference and competitor peptide}} - MF_{\text{no reference peptide}})/(MF_{\text{reference peptide}} - MF_{\text{no reference peptide}})] \times 100\%$. The binding affinity of competitor peptide is expressed as the concentration needed to inhibit 50% binding of the FL-labeled reference peptide (IC_{50}). An $IC_{50} \leq 5$ μM was considered high affinity binding, 5 μM $< IC_{50} \leq 15$ μM was considered intermediate affinity binding, 15 μM $< IC_{50} \leq 100$ μM was judged low affinity binding, and $IC_{50} > 100$ μM was considered not binding.

Peptide–MHC Complex Dissociation Assay. Binding stability at 37°C of peptides complexed with HLA-A*0201 was measured as described previously (56). In short, JY cells were treated with 10^{-4} M emetine (Sigma-Aldrich) for 1 h at 37°C to stop de novo synthesis of MHC class I molecules. Subsequently, endogenous bound peptides in HLA-A*0201 were removed by mild acid treatment (see above) and reconstituted with the peptide of interest at 200 μM in 2% FCS/IMDM containing 2 μg/ml human β_2-microglublin (Sigma-Aldrich) for 1.5 h at room temperature. Hereafter, cells were washed twice to remove free peptide and incubated at 37°C for 0, 2, 4, and 6 h. Subsequently, expressed HLA-A*0201–peptide complexes on JY cells were stained using the conformation-specific Moab BB7.2 and goat anti–mouse IgG–FITC and analyzed on a FACScan™ flow cytometer. The fluorescence index (FI) was calculated for each sample as: $(MF_{\text{sample}} - MF_{\text{background}})/MF_{\text{background}}$, where $MF_{\text{background}}$ is the value without peptide. The percentage of residual HLA-A*0201 molecules was calculated by equating for each peptide the FI of $t = 2$ h to 100% and then using the formula: % remaining $= (FI_{t=n}/FI_{t=2}) \times 100\%$. Because the dissociation of peptides from MHC is a linear process, the stability of the peptide–MHC complexes was measured as the time required for 50% of the molecules to decay (DT_{50}), starting from $t = 2$. By linear regression analysis of the sequential measurements plotted against the percentage of remaining HLA-A*0201 molecules, the DT_{50} was calculated. As a positive control, the known highly stable HBV nucleocapsid 18–27 epitope was used.

In Vitro Proteasome-mediated Digestions. 20S proteasomes were purified from a B-LCL cell line as described (57). This cell type is known to contain immunoproteasomes (58). High LMP2 and LMP7 content was confirmed by two-dimensional immunoblotting (data not shown). To assess kinetics, digestions were performed with different incubation periods. Peptides (27 mer, 20 μg) were incubated with 1 μg of purified proteasome at 37°C for 1, 4, and 24 h in 300 μl proteasome digestion buffer as described (44). TFA (30 μl) was added to stop the digestion and samples were stored at −20°C before mass spectrometric analysis.

Mass Spectrometry. Electrospray ionization mass spectrometry was performed on a hybrid quadrupole time of flight mass spectrometer, a Q-TOF (Micromass), equipped with an on-line nanoelectrospray interface (capillary tip, 20-μm internal diameter × 90-μm outer diameter) with an approximate flow rate of 250 nl/min. This flow was obtained by splitting of the 0.4 ml/min flow of a conventional high pressure gradient system, using an Acurate flow splitter (LC Packings). Injections were done with a dedicated micro/nano HPLC autosampler, the FAMOS (LC Packings), equipped with two additional valves for phase system switching experiments. Digestion solutions were diluted five times in water/methanol/acetic acid (95:5:1, vol/vol/vol), and 1 μl was trapped on the precolumn (MCA-300-05-C8; LC Packings) in water/methanol/acetic acid (95:5:1, vol/vol/vol). Washing of the precolumn was done for 3 min to remove the buffers present in the digests. Subsequently, the trapped analytes were eluted with a steep gradient going from 70% B to 90% B in 10 min, with a flow of 250 nl/min (A, water/methanol/acetic acid [95:5:1, vol/vol/vol]; B, water/methanol/acetic acid [10:90:1, vol/vol/vol]). This low elution rate allows for a few additional mass spectrometry (MS)/MS experiments if necessary during the same elution. Mass spectra were recorded from mass 50–2,000 daltons every second with a resolution of 5,000 full width/half maximum (FWHM). The resolution allows direct determination of the monoisotopic mass, also from multiple charged ions. In MS/MS mode, ions were selected with a window of 2 daltons with the first quadrupole and fragments were collected with high efficiency with the orthogonal time of flight mass spectrometer. The collision gas applied was argon (4×10^{-5} mbar), and the collision voltage ~30 V. The peaks in the mass spectrum were searched in the digested precursor peptide using the Biolynx/proteins software (Micromass) supplied with the mass spectrometer. The intensity of the peaks in the mass spectra was used to establish the relative amounts of peptides generated after proteasome digestion. The relative amounts of the peptides are given as a percentage of the total amount of peptide digested by the proteasome at the indicated incubation time.

RT-PCR Assay for PRAME Expression. Analysis of PRAME mRNA expression was determined by RT-PCR. Total cellular RNA was isolated with Trizol (GIBCO BRL) according to the manufacturer's procedure. RT reaction was performed on 5 μg of total RNA in a reaction volume of 25 μl with 5 μl of 5× reverse transcriptase buffer (Promega), 2.5 μl each of 10 mM deoxynucleotides (Amersham Pharmacia Biotech), 0.5 μg oligo dT_{15} primer, 25 U of RNAsin (Promega), and 15 U avian myeloblastosis virus (AMV) reverse transcriptase (Promega). The RT reaction was incubated at 42°C for 60 min, heat inactivated for 10 min at 70°C, and diluted two times with water. For PCR amplification, 1 μl of reverse transcribed cDNA reaction mixture was used as a template. PCR primers used for the analysis of PRAME expression were OPC 189 (sense primer, 5′-CTGTACTCATTTCCAGAGCCAGA-3′) and OPC 190 (antisense primer, 5′-TATTGAGAGGGTTTCCAAGGGGTT-3′; reference 48). PCR conditions were 5 min at 94°C followed by 34 cycles consisting of 30 s at 94°C, 2 min at 64°C, and 3 min at 72°C.

In Vitro CTL Response Induction and Generation of CTL Clones. PBMCs of two HLA-A*0201$^+$ healthy donors (one donor for induction against $PRA^{300–309}$ and the other donor for inductions against $PRA^{100–108}$, $PRA^{142–151}$, $PRA^{425–433}$, and $PRA^{47–56}$) were obtained by the Ficoll-Paque method and used for CTL in-

ductions. To optimally use all APCs present in PBMCs, we developed a culture system that yields a mix of activated B cells and mature DCs to be used as APCs during the primary induction step. PBMCs were separated in a T cell fraction and a fraction containing B cells and monocytes by SRBC rosetting. The T cell fraction was cryopreserved. The mixture of monocytes and B cells was cultured in 24-well plates at a concentration of 10^6 cells/well in complete culture medium containing 800 U/ml GM-CSF (provided by S. Osanto, Leiden University Medical Center), 500 U/ml IL-4 (PeproTech), and 500 ng/ml CD40 mAb (clone B-B20; Serotec) for 6 d. This culture system achieved a threefold effect: (a) GM-CSF and IL-4 induced differentiation of monocytes into immature DCs (59), (b) IL-4 and CD40 mAbs caused activation and proliferation of B cells (60), and (c) CD40 mAb mediated maturation of immature DCs (61). At day 3, cytokines and CD40 mAb were replenished. To further promote CTL-inducing capacity, the APC mix was cultured for an additional 2 d with 0.4 ng/ml LPS (Difco Labs), 500 U/ml IFN-γ (Boehringer Mannheim), and 500 ng/ml CD40 mAb. At day 8 the APC mix was pulsed with 50 μg/ml peptide (each peptide separately) for 4 h at room temperature, irradiated (30 Gy), and washed to remove free peptide. The cryopreserved autologous T cell fraction was thawed and depleted from $CD4^+$ T cells using magnetic beads (Dynal). The primary induction was performed in 96-well U-bottomed plates. APCs at a concentration of 10,000/well were cocultured with 50,000 $CD8^+$ T cells/well in culture medium containing 10% human pooled serum (HPS), 5 ng/ml IL-7 (PeproTech), and 0.1 ng/ml IL-12 (Sigma-Aldrich). At day 7 after initiation of induction, the CTL microcultures were harvested (pooled), washed, and restimulated at a concentration of 40,000 responder cells/well of 96-well U-bottomed plates in culture medium containing 10% HPS, 5 ng/ml IL-7, and 0.1 ng/ml IL-12. Autologous-activated B cells, generated via the protocol described by Schultze et al. (60), irradiated (75 Gy), and peptide pulsed (50 μg/ml) for 4 h at room temperature in culture medium containing 2% FCS and 3 μg/ml β_2-microglublin (Sigma-Aldrich) after mild acid elution to remove naturally presented peptides from the MHC class I molecules (see Materials and Methods, MHC binding assay), were used at a concentration of 10,000 cells/well as restimulator APCs. Restimulations were repeated at day 14 and 21 in a similar way, with the exception of IL-7 being replaced by 20 IU/ml IL-2 (Chiron Corp.). At day 29, the CTL bulk culture was cloned by standard limiting dilution procedures. CTL clones were maintained by aspecific stimulation every 7 to 12 d using a feeder mixture consisting of allogeneic PBMCs and B-LCL in culture medium containing 10% FCS, 1.5% leucoagglutinin (Sigma-Aldrich), and 240 IU/ml IL-2.

^{51}Cr Cytotoxicity Assay, HLA Class I Blocking, and Proteasome Inhibition. CTL activity was measured in standard chromium release assays. In brief, after ^{51}Cr labeling (1 h), target cells (2,000/well) were added to various numbers of effector cells in a final volume of 100 μl complete culture medium in 96-well U-bottomed plates. After 4 h incubation at 37°C, supernatants were harvested. The mean percentage of specific lysis of triplicate wells was calculated according to: (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100%. For peptide titration experiments, ^{51}Cr-labeled target cells (2,000/well) were pulsed with titrated amounts of peptide for 1 h at 37°C in 96-well plates. Subsequently, CTLs were added at an E/T ratio of 10. HLA class I blocking was accomplished by treatment of 2×10^5 ^{51}Cr-labeled M453 melanoma cells for 1 h with mAb W6.32 or control IgG2a at room temperature before addition to effector cells. Inhibition of proteasome function in melanoma FM3 was performed by treatment with 10 μM of lactacystin (Calbiochem) for 17 h during culture at 37°C. Thereafter, FM3 cells were harvested and ^{51}Cr labeled for use in the cytotoxicity assay. Reconstitution of lysis by peptide was performed by pulsing lactacystin-treated and ^{51}Cr-labeled FM3 cells for 30 min with 5 μM peptide.

Results

*Identification of HLA-A*0201 Binding Peptides from PRAME.* To select candidate HLA-A*0201 binding peptides from PRAME, its aa sequence was screened for HLA-A*0201 binding motif containing peptides with a combination of two known binding prediction algorithms (62, 63). Only peptides of 9 or 10 aa length were included, taking into account the low prevalence of HLA-A*0201–restricted CTL epitopes of 8 or 11 aa length (64). In total, 128 peptides (65 nonamers and 63 decamers) were synthesized in order to determine their actual binding affinity for HLA-A*0201 using a competition-based cellular binding assay (54). 19 high affinity binding peptides were identified ($IC_{50} \leq 6$ μM), and 27 peptides bound with intermediate affinity (6 μM $< IC_{50} \leq 15$ μM), whereas the other peptides displayed a low (15 μM $< IC_{50} \leq 100$ μM) or undetectable binding capacity ($IC_{50} > 100$ μM; Table I). To more precisely define binding characteristics, peptide–MHC stability was assessed by measuring the dissociation rate of high affinity binding peptides complexed with HLA-A*0201 at 37°C (56). Two of the tested high affinity binding peptides ($PRA^{292–301}$ and $PRA^{190–199}$) showed a high off rate from HLA-A*0201, because <10% of HLA-A*0201–peptide complexes were detectable after 2 h incubation at 37°C. In previous work we have detected a strong correlation between MHC–peptide binding stability and immunogenicity in vivo (56). Therefore, $PRA^{292–301}$ and $PRA^{190–199}$ are, with respect to their binding characteristics, not likely to be efficiently presented in HLA-A*0201. For all other peptides, the 50% decay time (DT_{50}) was 2.5 h or longer (Table II), indicating a stable association with HLA-A*0201.

*In Vitro Proteasome-mediated Digestions of 27-mer Polypeptides Encompassing HLA-A*0201 Binding Peptides.* The two most important requirements for a peptide to be naturally presented as CTL epitope are: (a) proper excision from the protein by the proteolytic machinery and (b) sufficient binding affinity for HLA class I molecules. Therefore, we analyzed in vitro proteasome-mediated digestions of 27-mer polypeptides encompassing the 19 identified high affinity HLA-A*0201 binding peptides. Potential epitopes were primarily assessed for efficient liberation (i.e., by a major cleavage site at 1 h incubation) of their precise COOH terminus, which is a first requirement for the generation of most CTL epitopes (34–37). Intactness of the candidate epitope was evaluated as a secondary factor favoring efficient processing and presentation. 20S proteasomes isolated from a human EBV–transformed B cell line were used for digestions with 1-, 4-, and 24-h incubation periods, and mass spectrometry profiles of the digestion products were analyzed. Digestion patterns of four 27-mer

Table I. *Binding Affinity for HLA-A*0201 of 128 Nonamers and Decamers Derived from PRAME*

Start*	Sequence	Length	IC_{50}‡	Start	Sequence	Length	IC_{50}	Start	Sequence	Length	IC_{50}
300	ALYVDSLFFL	10	1.7	466	RLRELLCEL	9	14.0	86	LMKGQHLHL	9	62.3
142	SLYSFPEPEA	10	1.9	33	SLLKDEALAI	10	14.0	240	VTCTWKLPTL	10	67.2
47	LLPRELFPPL	10	2.1	422	ALQSLLQHL	9	14.2	44	ALELLPREL	9	71.3
435	NLTHVLYPV	9	2.5	103	GLDVLLAQEV	10	15.2	379	TLQDLVFDEC	10	71.9
292	FLSLQCLQAL	10	2.5	231	QLDSIEDLEV	10	15.5	371	ALLERASAT	9	72.0
394	QLLALLPSL	9	2.9	312	RLDQLLRHV	9	15.7	353	VLSLSGVMLT	10	74.6
182	FLKEGACDEL	10	3.0	493	RTFYDPEPI	9	15.8	305	SLFFLRGRL	9	79.4
294	SLQCLQALYV	10	3.2	308	FLRGRLDQL	9	16.1	409	TTLSFYGNSI	10	80.8
422	ALQSLLQHLI	10	3.2	429	HLIGLSNLT	9	16.3	93	HLETFKAVL	9	89.0
425	SLLQHLIGL	9	3.7	85	VLMKGQHLHL	10	17.0	319	HVMNPLETL	9	90.3
258	QMINLRRLLL	10	4.0	316	LLRHVMNPL	9	17.4	18	SVWTSPRRLV	10	>100
190	ELFSYLIEKV	10	4.5	353	VLSLSGVML	9	17.4	20	WTSPRRLVEL	10	>100
248	TLAKFSPYL	9	4.6	172	FIPVEVLVDL	10	17.5	26	LVELAGQSL	9	>100
39	ALAIAALEL	9	5.1	134	TVWSGNRASL	10	18.4	51	ELFPPLFMA	9	>100
100	VLDGLDVLL	9	5.2	339	VMHLSQSPSV	10	18.5	67	QTLKAMVQA	9	>100
333	RLSEGDVMHL	10	5.4	72	MVQAWPFTC	9	18.5	70	KAMVQAWPFT	10	>100
462	YLHARLREL	9	5.4	390	ITDDQLLALL	10	18.9	78	FTCLPLGVL	9	>100
360	MLTDVSPEPL	10	5.6	18	SVWTSPRRL	9	19.1	84	GVLMKGQHL	9	>100
419	SISALQSLL	9	5.7	315	QLLRHVMNPL	10	19.7	95	ETFKAVLDGL	10	>100
432	GLSNLTHVL	9	6.8	71	AMVQAWPFT	9	20.0	133	WTVWSGNRA	9	>100
214	KIFAMPMQDI	10	7.2	207	RLCCKKLKI	9	20.8	155	MTKKRKVDGL	10	>100
320	VMNPLETLSI	10	8.6	247	PTLAKFSPYL	10	21.1	165	STEAEQPFI	9	>100
39	ALAIAALELL	10	9.0	219	PMQDIKMIL	9	23.9	180	DLFLKEGAC	9	>100
390	ITDDQLLAL	9	9.2	459	RLAYLHARL	9	24.3	198	KVKRKKNVL	9	>100
242	CTWKLPTLA	9	9.3	264	RLLLSHIHA	9	24.6	205	VLRLCCKKL	9	>100
99	AVLDGLDVLL	10	9.4	217	AMPMQDIKMI	10	24.6	222	DIKMILKMV	9	>100
308	FLRGRLDQLL	10	9.6	361	LTDVSPEPL	9	26.8	224	KMILKMVQL	9	>100
355	SLSGVMLTDV	10	9.9	430	LIGLSNLTHV	10	27.2	234	SIEDLEVTC	9	>100
34	LLKDEALAI	9	10.2	33	SLLKDEALA	9	29.2	234	SIEDLEVTCT	10	>100
284	YIAQFTSQFL	10	10.4	258	QMINLRRLL	9	31.2	237	DLEVTCTWKL	10	>100
71	AMVQAWPFTC	10	10.4	91	HLHLETFKAV	10	31.8	240	VTCTWKLPT	9	>100
470	LLCELGRPSM	10	10.5	297	CLQALYVDSL	10	33.8	261	NLRRLLLSHI	10	>100
186	GACDELFSYL	10	10.6	372	LLERASATL	9	35.0	325	ETLSITNCRL	10	>100
410	TLSFYGNSI	9	11.0	401	SLSHCSQLT	9	36.9	368	PLQALLERA	9	>100
25	RLVELAGQSL	10	11.1	397	ALLPSLSHC	9	42.6	382	DLVFDECGI	9	>100
91	HLHLETFKA	9	11.1	389	GITDDQLLAL	10	47.3	382	DLVFDECGIT	10	>100
100	VLDGLDVLLA	10	11.9	417	SISISALQSL	10	48.2	383	LVFDECGIT	9	>100
454	TLHLERLAYL	10	12.2	259	MINLRRLLL	9	48.2	389	GITDDQLLA	9	>100
371	ALLERASATL	10	12.9	479	MVWLSANPC	9	49.0	401	SLSHCSQLTT	10	>100
326	TLSITNCRL	9	13.2	160	KVDGLSTEA	9	51.2	473	ELGRPSMVWL	10	>100
462	YLHARLRELL	10	13.3	436	LTHVLYPVPL	10	53.0	481	WLSANPCPHC	10	>100
350	QLSVLSLSGV	10	13.3	226	ILKMVQLDSI	10	56.1	493	RTFYDPEPIL	10	>100
99	AVLDGLDVL	9	13.4	292	FLSLQCLQA	9	58.7				

*Position in PRAME of the NH_2-terminal aa of the peptide. Peptides are listed in order of their IC_{50}.
‡IC_{50} is peptide concentration needed to inhibit binding of FL-labeled reference peptide for 50% (IC_{50} in μM).

polypeptides, all containing potential high affinity HLA-A*0201 binding epitopes, are shown in Fig. 1.

Fig. 1 A shows the digestions of PRAME 90–116, which harbors five HLA-A*0201 binding peptides (Table I) with their natural flanking residues. Of the COOH-terminal residues of the five HLA-A*0201 binding peptides, only Leu-108 was generated (fragments containing this COOH terminus added up to 8% at 1 h digestion). Therefore, both the 9-mer VLDGLDVLL ($PRA^{100–108}$) and the 10-mer AVLDGLDVLL ($PRA^{99–108}$) represent potential CTL epitopes. The NH_2 terminus of the epitope precursor is likely to be Lys-98, because the fragments most frequently generated were aa 90–97 and its complement aa 98–116, indicating an abundantly cleaved site after Phe-97.

Fig. 1 B shows the digestions of 27-mer PRAME 133–159, which contains 10-mer SLYSFPEPEA ($PRA^{142–151}$), the second best HLA-A*0201 binding peptide (Table I). Fragments sharing Ala-151 as COOH terminus added up to 29% at 1 h digestion, indicating an abundantly cleaved site after this residue. Furthermore, fragment aa 142–159 and the complementary fragment aa 133–141 were abundantly present, pointing to a major cleavage site after Ala-141. Thus, major cleavage sites were present just after and before SLYSFPEPEA, rendering this peptide a potential efficiently generated CTL epitope.

Table II. *Stability of High Affinity Binding Peptides in HLA-A*0201*

Start*	Sequence‡	Affinity IC_{50}§	Stability DT_{50}‖
		μM	*h*
300	ALYVDSLFFL	1.7	>4
142	SLYSFPEPEA	1.9	3
47	LLPRELFPPL	2.1	2.5
435	NLTHVLYPV	2.5	3
292	FLSLQCLQAL	2.5	N.S.¶
394	QLLALLPSL	2.9	>4
182	FLKEGACDEL	3.0	3
294	SLQCLQALYV	3.2	>4
422	ALQSLLQHLI	3.2	2.5
425	SLLQHLIGL	3.7	>4
258	QMINLRRLLL	4.0	>4
190	ELFSYLIEKV	4.5	N.S.
248	TLAKFSPYL	4.6	>4
100	VLDGLDVLL	5.2	2.5
	HBV control**		>4

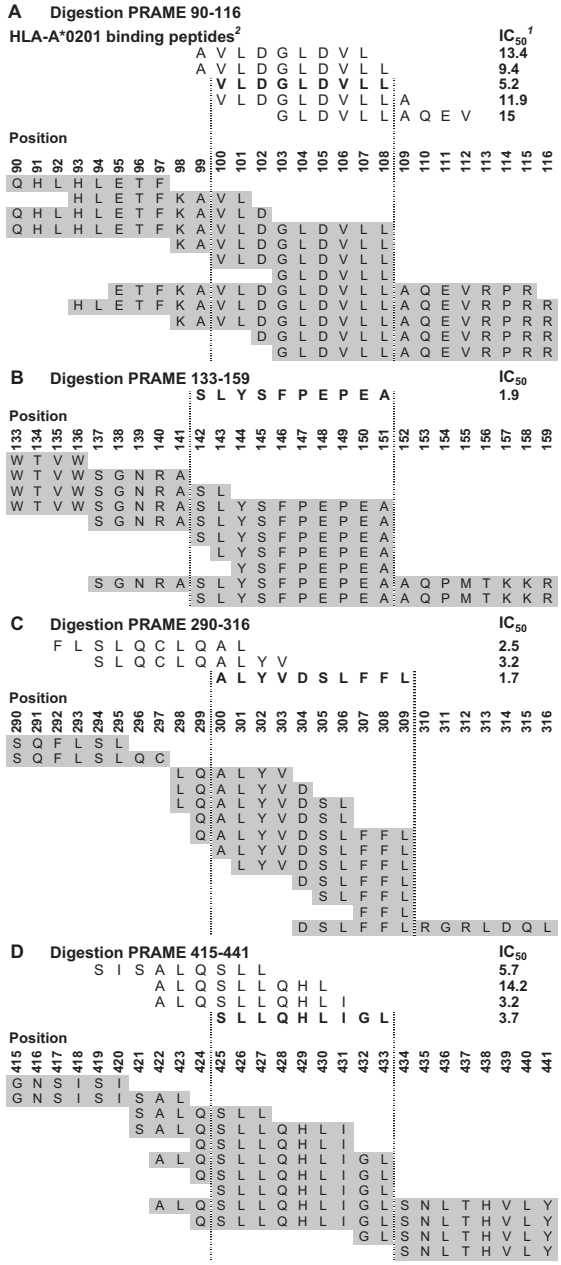
*Start aa position of peptide in PRAME.
‡In addition to all high affinity binding peptides, PRAME 100–108 and 371–380 are tested.
§Binding affinity expressed as IC_{50} (see Table I).
‖DT_{50} is given starting from $t = 2$ h at 37°C.
¶N.S., not stable; <10% of HLA molecules detectable after 2 h incubation at 37°C.
**HBV nucleocapsid 18–27 is used as control peptide.

Fig. 1 C depicts digestions of PRAME 290–316 encompassing 10-mer ALYVDSLFFL ($PRA^{300–309}$), which bound best in HLA-A*0201 (Table I) and has its COOH terminus (Leu-309) in common with the already described HLA-A24–presented 9-mer LYVDSLFFL ($PRA^{301–309}$; reference 48). As might be expected on that basis, a cleavage site after Leu-309 was observed, because digestion fragments sharing this COOH terminus were abundantly generated after 1 h incubation. However, $PRA^{300–309}$ itself was found intact only after 24 h incubation at low quantities. This is probably due to cleavage sites within this potential epitope (after Val-303, Asp-304, and Leu-306). HLA-A*0201 binding peptides $PRA^{292–301}$ and $PRA^{294–303}$ (also in PRAME 290–316) were, respectively, not COOH-terminally generated and not found as intact fragment, indicating that these peptides are not likely to be naturally generated in the processing pathway.

Fig. 1 D shows the digestion pattern of PRAME 415–441, which harbors four peptides binding in HLA-A*0201 (Table I). The NH_2-terminally elongated decameric precursor $_{424}$QSLLQHLIGL$_{433}$ of high affinity binding 9-mer SLLQHLIGL ($PRA^{425–433}$) was efficiently generated. The abundant generation of the COOH-terminal and NH_2-terminal counterparts of this 10-mer precursor (fragments aa 434–441 and aa 415–423, respectively) were also pointing to major cleavage sites just after and before $_{424}$QSLLQHLIGL$_{433}$, indicating $PRA^{425–433}$, a potential CTL epitope. The three other HLA-A*0201 binding peptides were either not COOH-terminally excised ($PRA^{419–427}$ and $PRA^{422–430}$) or the correct COOH terminus was found only after 4 h incubation ($PRA^{422–431}$).

A concise representation of digestion analysis of 27-mers harboring all 19 high affinity binding peptides, including those discussed above, is shown in Fig. 2. Summarizing, 11 HLA-A*0201 binding peptides were either not COOH-terminally excised ($PRA^{47–56}$, $PRA^{435–443}$, $PRA^{292–301}$, $PRA^{182–191}$, $PRA^{248–256}$, $PRA^{100–108}$, and $PRA^{360–369}$) or the correct COOH terminus was generated only after 4 h incubation by a minor cleavage site ($PRA^{394–402}$, $PRA^{422–431}$, $PRA^{190–199}$, and $PRA^{419–427}$). The absence or late appearance of fragments containing the correct COOH terminus render these 11 peptides very unlikely to constitute naturally processed epitopes. Furthermore, three peptides were COOH-terminally liberated at 1 h digestion but only in very low quantities (<1%, data not shown; $PRA^{258–267}$, $PRA^{333–342}$, and $PRA^{462–470}$), whereas $PRA^{294–303}$ was not found as intact fragment. Consequently, it is doubtful that the latter peptides are efficiently generated in vivo. Only the high affinity binding peptides $PRA^{100–108}$, $PRA^{142–151}$, $PRA^{300–309}$, and $PRA^{425–433}$ were COOH-terminally excised by a major cleavage site at 1 h incubation and found intact in digestion fragments, indicating possible CTL epitopes (Figs. 1 and 2). Therefore, these four peptides were chosen for CTL inductions.

*In Vitro Human CTL Inductions against Four Putative HLA-A*0201–restricted Epitopes.* Separate CTL inductions, using PBMCs of healthy donors, were performed against VLDGLDVLL ($PRA^{100–108}$), SLYSFPEPEA



Fragment[3,4]		Intensity (% digested)[5]		
Start	End	1 h	4 h	24 h
90	97	26	19	29
93	101	8	2	3
90	102	5	2	2
90	108	2	1	0
98	108	2	1	3
100	108	0	0.1	0.2
103	108	4	1	2
95	115	3	4	3
93	116	7	31	18
98	116	29	28	27
102	116	8	5	6
103	116	7	5	6

Fragment		Intensity (% digested)		
Start	End	1 h	4 h	24 h
133	136	22	35	26
133	141	21	12	24
133	143	5	4	3
133	151	18	11	10
137	151	4	3	5
142	151	4	2	6
143	151	2	2	3
144	151	1	1	1
137	159	10	23	11
142	159	12	7	12

Fragment		Intensity (% digested)		
Start	End	1 h	4 h	24 h
290	295	40	45	32
290	297	4	4	1
298	303	5	4	6
298	304	5	4	5
298	306	2	2	2
299	306	4	3	4
299	309	0	0	0.4
300	309	0	0	0.3
301	309	0	0	0.7
304	309	4	5	9
305	309	8	9	24
307	309	21	19	13
304	316	7	5	3

Fragment		Intensity (% digested)		
Start	End	1 h	4 h	24 h
415	420	13	12	6
415	423	17	15	14
421	427	0	2	1
421	431	0	4	2
424	431	0	8	3
422	433	0	1	7
424	433	17	10	8
425	433	0	1	2
422	441	0	0	8
424	441	0	3	3
432	441	0	5	3
434	441	54	39	42

Figure 1. In vitro proteasome-mediated digestions of four 27-mer PRAME polypeptides containing potential HLA-A*0201–restricted epitopes. 20S proteasomes isolated from an EBV-transformed B cell line were coincubated with 27-mer PRAME peptides at 37°C for the indicated time points. Digestion mixtures were analyzed by mass spectrometry as described in Materials and Methods. Generated digestions fragments are depicted as shaded sequences. The digestion of 27-mer PRAME 90–116 is represented in A, digestion of PRAME 133–159 is depicted in B, in C the digestion of PRAME 290–316 is showed, and D represents the digestion of PRAME 415–441. Notes: (1) IC_{50} as determined in competition binding assay (see Table I); (2) peptides binding with high or intermediate affinity to HLA-A*0201 are shown. Predicted epitopes used for CTL induction are printed in bold; (3) digestion fragments are shaded and ordered according to their COOH terminus; (4) digestion fragments generated for <1% at 1 h digestion or <3% at 4 and 24 h incubation and not relevant for epitope prediction are not shown; (5) intensity is expressed as percentage of total summed mass-peak intensities of digested 27-mer at the indicated incubation time.

(PRA$^{142–151}$), ALYVDSLFFL (PRA$^{300–309}$), and SLLQHLIGL (PRA$^{425–433}$) according to the protocol described in Materials and Methods. At day 28, the CTL bulk cultures were tested in a ^{51}Cr release assay to asses peptide specificity. The CTL cultures raised against SLYSFPEPEA and SLLQHLIGL showed high specificity for targets loaded with the inducing peptide (at an E/T ratio of 5, both cultures reached 80% lysis compared with 20% lysis of targets loaded with a control peptide), whereas the other two CTL cultures displayed only slightly increased lysis of targets loaded with the relevant peptide (data not shown). The four CTL bulk cultures were cloned by limiting dilution at day 29. The peptide specificity of generated CTL clones was initially assessed in a split-well cytotoxicity assay. Despite low peptide specificity of CTL bulk cultures against VLDGLDVLL and ALYVDSLFFL, CTL clones specific for

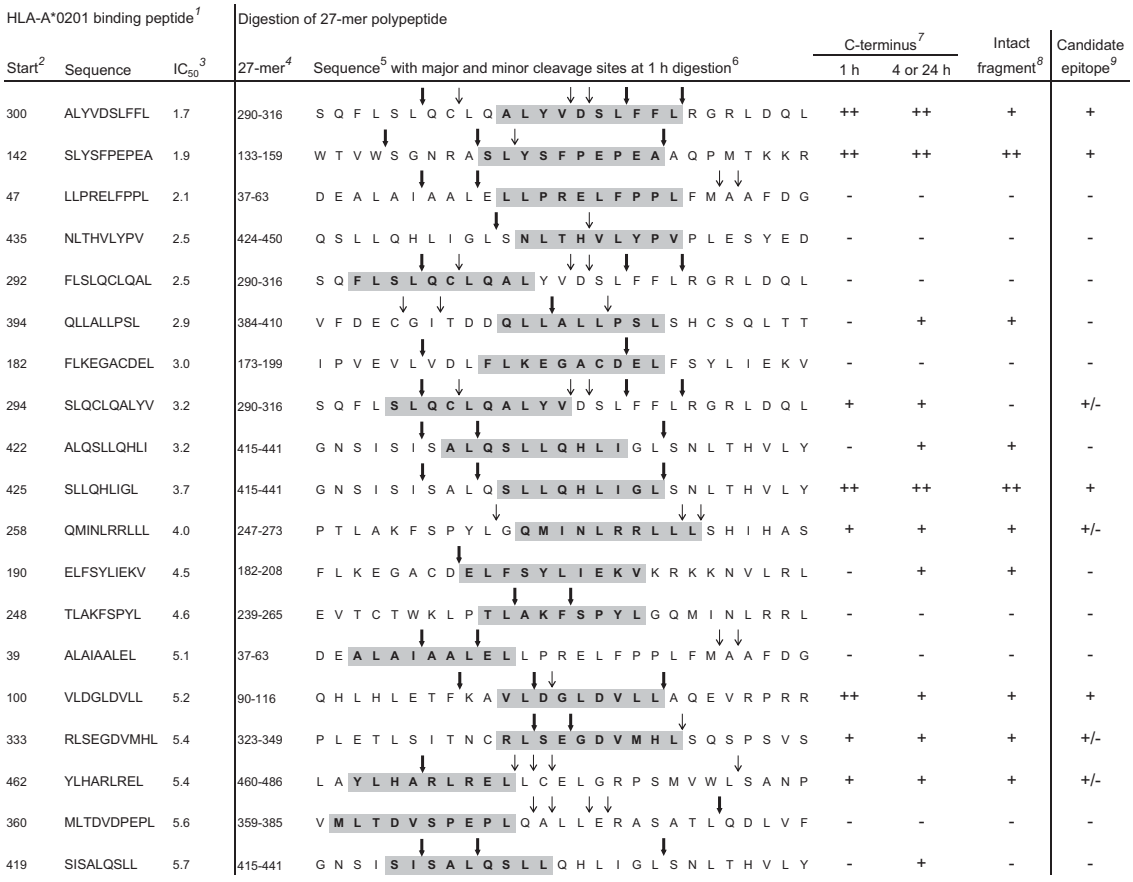


Figure 2. Proteasome-mediated cleavage patterns of 27-mer peptides encompassing 19 high affinity HLA-A*0201 binding PRAME peptides. 20S proteasomes isolated from an EBV-transformed B cell line were coincubated with 27-mer PRAME peptides at 37°C for the indicated time points. Digestion mixtures were analyzed by mass spectrometry as described in Materials and Methods. Major and minor cleavages sites at 1 h digestion are depicted. Notes: (1) all 19 high affinity binding peptides ($IC_{50} < 6$ μM) are listed and ranked according to their binding affinity for HLA-A*0201; (2) start aa position in PRAME of the HLA-A*0201 binding peptide. (3) IC_{50} (in μM) as determined in competition binding assay (see Table I); (4) start and end aa position in PRAME of 27-mer polypeptide encompassing the high affinity binding peptide; (5) aa sequence of 27-mer polypeptide (aa sequence of HLA-A*0201 binding peptide is printed in bold and shaded); (6) major (bold arrows) and minor (thin arrows) cleavage sites at 1 h incubation are depicted, classified according to the following definitions. Major site: fragments containing as COOH terminus the residue NH_2-terminal from the cleavage are present for ≥5% at 1 h incubation. Minor site: fragments containing as COOH terminus the residue NH_2-terminal from the cleavage are present for <5% at 1 h incubation. (7) Generation by digestion of fragments containing the correct COOH terminus of the HLA-A*0201 binding peptide. Generation at 1 h digestion or after a longer incubation period is separately indicated. Classification: (++) present for ≥5%, (+) present for <5%, (−) no fragments containing the correct COOH terminus were found. (8) Generation by digestion of fragments containing the intact HLA-A*0201 binding peptide and/or NH_2-terminal elongated precursors of the peptide. Classification: (++) present for ≥5% at 1 h incubation, (+) present for <5% at 1 h or only detectable after 4 or 24 h, (−) no fragments containing the HLA-A*0201 binding peptide were found. (9) Epitope prediction based on digestion results. Classification: (+) most likely an epitope, (+/−) doubtful epitope, (−) not an epitope.

all four peptides were found. In summary, 51 of 576 (9%) CTL clones induced against VLDGLDVLL ($PRA^{100–108}$) showed specific lysis of peptide pulsed targets and 19 of 202 (9%) CTL clones raised against ALYVDSLFFL ($PRA^{300–309}$) displayed peptide specificity. As may be expected, higher percentages, namely 92% (279 of 304), of clones against SLYSFPEPEA ($PRA^{142–151}$) and 29% (97 of 336) of clones against SLLQHLIGL ($PRA^{425–433}$) showed peptide specificity. Based on peptide reactivity and growth characteristics, several CTL clones were functionally characterized in detail. For each specificity, one CTL clone is presented in this study. As shown in Fig. 3 A, representative CTL clones raised against the four different peptides all showed highly specific and efficient lysis of T2 cells pulsed with 5 μM of their inducing peptides at low E/T ratios. Peptide sensitivity of the CTL clones was determined in peptide titration experiments (Fig. 3 B). CTL no. 551 (anti-$PRA^{100–108}$) was able to half-maximally lyse targets loaded with VLDGLD-

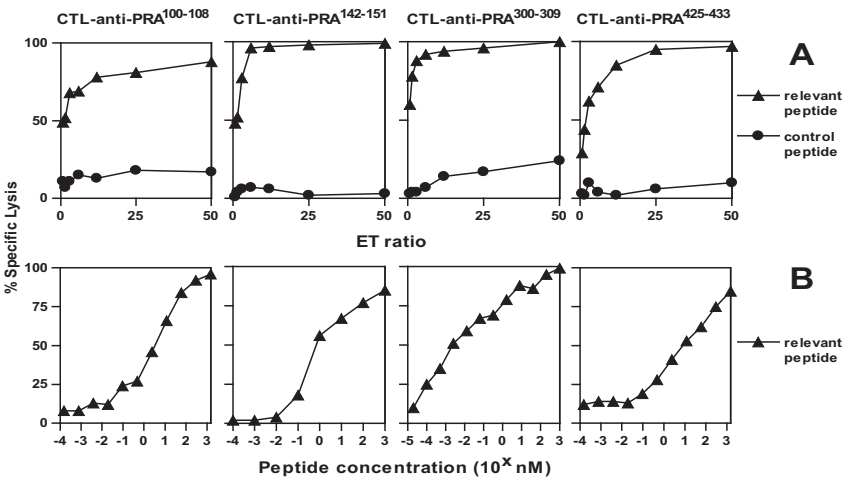


Figure 3. HLA-A*0201–restricted peptide specificity and sensitivity of CTL clones raised against four PRAME peptides. (A) Lysis by CTL clones no. 551 anti-PRA$^{100–108}$, no. 314 anti-PRA$^{142–151}$, no. 460 anti-PRA$^{300–309}$, and no. 1257 anti-PRA$^{425–433}$ of ^{51}Cr-labeled T2 cells loaded with 5 μM of the relevant peptide (▲) vs. an irrelevant HLA-A*0201 binding peptide (●) at different E/T ratios ranging from 50 to 0.75. (B) Lysis by the same set of CTL clones of ^{51}Cr-labeled T2 cells pulsed for 1 h with titrated concentrations of relevant peptide (▲). The CTL clones were used at an E/T ratio of 10. Results of one representative experiment out of three performed are shown.

VLL at ~5 nM peptide. CTL no. 314, raised against SLYSFPEPEA (PRA$^{142–151}$), lysed T2 cells at half-maximal level when pulsed with <1 nM of the inducing peptide. CTL no. 460 (anti-PRA$^{300–309}$) was extremely sensitive in lysing T2 cells pulsed with ALYVDSLFFL: half-maximal lysis was reached at ~3 pM peptide concentration. Finally, CTL no. 1257 (anti-PRA$^{425–433}$) was able to half-maximally lyse targets loaded with SLLQHLIGL at <12 nM. To analyze clonality of the CTL clones under investigation, we performed RT-PCR analysis with a panel of 24 primers of junctional regions of TCRB transcripts from 22 well-established TCRBV families to determine Vβ usage of the TCR (65). All CTL clones were shown to use a single Vβ, confirming clonality of the clones (data not shown).

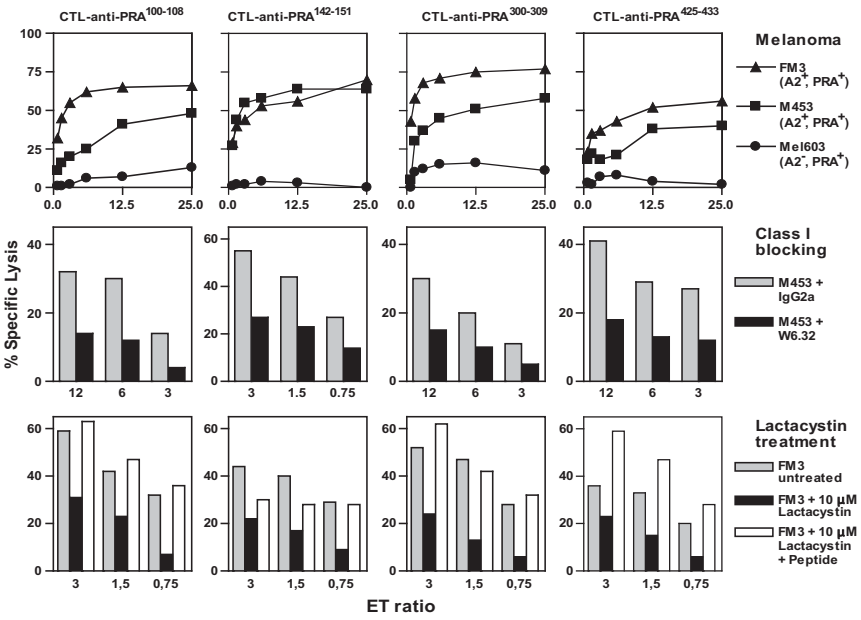


Figure 4. Recognition by CTL clones of four endogenously processed PRAME epitopes presented on melanoma cell lines in an HLA class I–restricted and proteasome-dependent fashion. (Top) Lysis of ^{51}Cr-labeled melanoma cell line Mel603, expressing PRAME but lacking HLA-A*0201 expression (●), was tested vs. lysis of M453 (■) and FM3 (▲), both expressing PRAME and HLA-A*0201 together. CTL clones no. 551 anti-PRA$^{100–108}$, no. 314 anti-PRA$^{142–151}$, no. 460 anti-PRA$^{300–309}$, and no. 1257 anti-PRA$^{425–433}$ were used at E/T ratios ranging from 25 to 0.75. (Middle) Lysis of ^{51}Cr-labeled M453 was tested after 1 h preincubation with HLA class I blocking mAb W6.32 (black bars) or an IgG2a control Ab (gray bars). (Bottom) Lysis of ^{51}Cr-labeled FM3 was tested after 17 h treatment with 10 μM of the proteasome inhibitor lactacystin (black bars) or without treatment (gray bars). As control, the lactacystin-treated cells were loaded with the relevant peptide (white bars). Results of one representative experiment of at least three performed are shown.

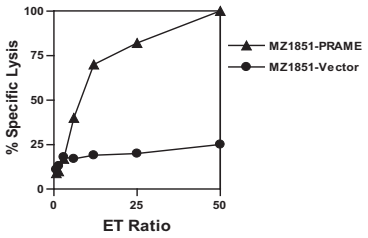


Figure 5. Lysis of PRAME transfected renal cell carcinoma cell line MZ1851 by CTL anti-PRA$^{300–309}$. CTL no. 460 directed against PRA$^{300–309}$ was tested on ^{51}Cr-labeled MZ1851 (HLA-A*0201^{+} but lacking PRAME expression) transfected with PRAME cDNA (▲) vs. MZ1851 transfected with the empty vector (●). CTL no. 460 was used at E/T ratios ranging from 50 to 0.75. Results of one representative experiment of three performed are shown.

*CTLs Raised against Four PRAME Peptides Recognize Melanoma Cell Lines Coexpressing HLA-A*0201 and PRAME in an HLA I–restricted and Proteasome-dependent Fashion.* Endogenous presentation of the candidate epitopes PRA$^{100–108}$, PRA$^{142–151}$, PRA$^{300–309}$, and PRA$^{425–433}$ in HLA-A*0201 was explored by assessing the ability of CTL clones that were raised against these peptides to specifically lyse melanoma cell lines M453 and FM3 expressing HLA-A*0201 (confirmed by flow cytometry, data not shown) and PRAME (determined by Northern blotting, data not shown). Both melanoma cell lines were efficiently lysed by all four CTL clones as measured in a ^{51}Cr release assay, whereas the melanoma Mel603 expressing PRAME (assayed with RT-PCR, data not shown) but lacking HLA-A*0201 was not killed above background level (Fig. 4, top panel). Lysis of M453 was significantly inhibited after treatment of this target with HLA class I blocking mAb W6.32 (Fig. 4, middle panel), indicating that killing of M453 by these CTL clones involved HLA class I–restricted recognition. Furthermore, proteasome inhibition experiments with lactacystin were performed. Lysis of FM3 pretreated for 17 h with lactacystin (10 μM) was significantly diminished (Fig. 4, bottom panel). This indicated, in concordance with our in vitro proteasome digestion data, that generation of the four epitopes is proteasome dependent. To confirm PRAME as source of antigen naturally presented in HLA-A*0201, renal cell carcinoma cell line MZ1851, which is HLA-*0201^{+} but lacks PRAME expression, was transfected with full-length PRAME cDNA (MZ1851-PRAME). As shown in Fig. 5, PRAME expression (confirmed by RT-PCR) sensitized MZ1851-PRAME for lysis by a CTL clone directed against PRA$^{300–309}$.

*CTLs Reactive with Four PRAME Epitopes Lyse a Broad Array of Tumor Cell Lines Expressing HLA-A*0201 and PRAME.* To investigate HLA-A*0201–restricted presentation of PRA$^{100–108}$, PRA$^{142–151}$, PRA$^{300–309}$, and PRA$^{425–433}$ on tumor cells from histologic origins other than melanoma, we used panels of cell lines derived from various tu-

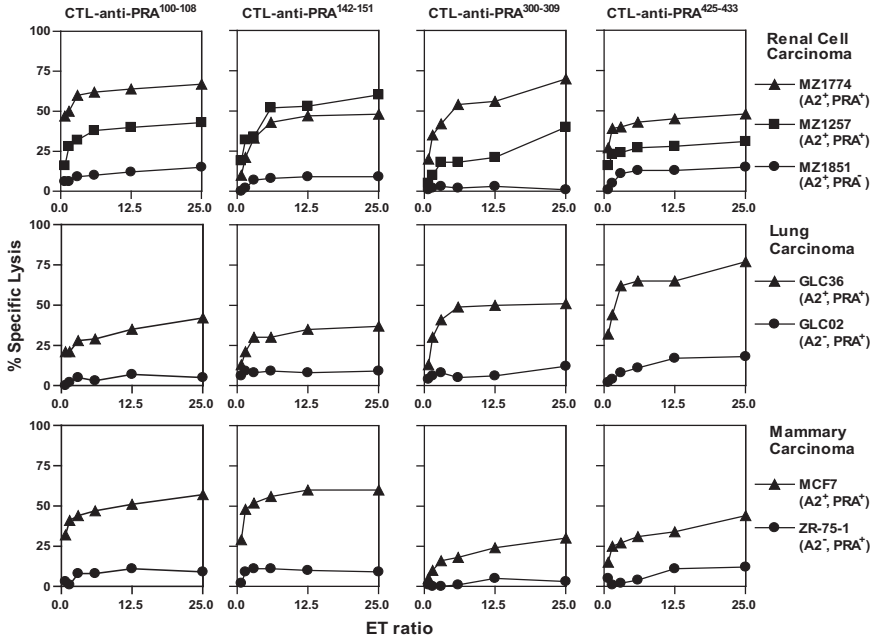


Figure 6. Lysis of tumor cell lines from multiple histologic origins by CTL anti-PRAME in a PRAME-specific and HLA-A*0201–restricted fashion. (Top) Lysis of ^{51}Cr-labeled renal cell carcinoma cell lines MZ1851, expressing HLA-A*0201 but PRAME negative (●), MZ1257 (PRAME^{+} and HLA-A*0201^{+}) (■), and MZ1774 (PRAME^{+} and HLA-A*0201^{+}) (▲) was compared. (Middle) Lysis of ^{51}Cr-labeled lung carcinoma cell lines. GLC02, expressing PRAME but HLA-A*0201 negative (●) and GLC36 (▲) expressing both PRAME and HLA-A*0201 was compared. (Bottom) ^{51}Cr-labeled mammary carcinoma cell lines MCF7 (HLA-*0201^{+} and PRAME^{+}) (●) and ZR-75-1, expressing PRAME but lacking HLA-A*0201 (▲), were tested. The CTL clones no. 551 anti-PRA$^{100–108}$, no. 314 anti-PRA$^{142–151}$, no. 460 anti-PRA$^{300–309}$, and no. 1257 anti-PRA$^{425–433}$ were used at E/T ratios ranging from 25 to 0.75. Results of one representative experiment of at least three performed are shown.

mor types which have been reported to express PRAME (48, 50).

Lysis by the selected CTL clones of tumor cell lines with or without HLA-A*0201 expression and naturally expressing PRAME or lacking PRAME expression was compared. HLA-A*0201 expression was confirmed by flow cytometry (data not shown) and PRAME expression by RT-PCR or Northern blotting (data not shown). Lysis of the HLA-A*0201$^+$ renal cell carcinoma (RCC) cell line MZ1851, which lacks PRAME expression, was compared with lysis of RCC cell lines MZ1257 and MZ1774, both expressing HLA-A*0201 and PRAME. The CTL clones reactive against the four different PRAME peptides showed significant lysis of the two PRAME$^+$ cell lines but not of MZ1851, again confirming PRAME as the source of target antigens (Fig. 6, top panel). Likewise, lysis of lung carcinoma cell lines was HLA-A*0201 restricted and PRAME specific, because only GLC36 expressing HLA-A*0201$^+$ and PRAME$^+$, and not GLC02, which is PRAME$^+$ but lacks HLA-A*0201 expression, was killed (Fig. 6, middle panel). Mammary carcinoma cell line MCF7 (HLA-A*0201$^+$ and PRAME$^+$) was killed efficiently as well, whereas cell line ZR-75-1, which lacks HLA-A*0201 but expresses PRAME, was not lysed above background level (Fig. 6, bottom panel). Finally, HPV16$^+$ cervix carcinoma cell line C33 and osteosarcoma cell line SAOS, both HLA-A*0201$^+$ and PRAME$^+$, were efficiently killed by the CTL clones (data not shown), underscoring the broad expression pattern of the PRAME epitopes by tumors. Taken together, we observed a consistent lysis of tumor cell lines when both the relevant MHC molecule and the tumor Ag were expressed. These results indicate that PRA$^{100–108}$, PRA$^{142–151}$, PRA$^{300–309}$, and PRA$^{425–433}$ are presented by HLA-A*0201 on a broad array of tumor cell lines.

*CTL Clones Recognizing a Nonprocessed High Affinity Binding Peptide Do Not Lyse PRAME and HLA-A*0201–expressing Tumor Cell Lines.* To further validate our epitope prediction procedure and investigate possible false negative epitope prediction, CTLs were induced against the highest affinity HLA-A*0201 binding peptide that was not generated in vitro by proteasome-mediated breakdown: LLPRELFPPL (PRA$^{47–56}$; Table I and Fig. 2). Digestion of the 27-mer PRAME 37–63 showed that the COOH terminus of LLPRELFPPL was not generated. Instead, cleavage sites were observed after Leu-45, Met-58, and Ala-59 (Fig. 2). Dual cleavage fragments $_{46}$ELLPRELFPPLFM$_{58}$ and $_{46}$ELLPRELFPPLFMA$_{59}$ were abundantly found at 1 h digestion (data not shown). Using the same induction protocol as used for the four identified epitopes, CTLs were generated against LLPRELFPPL. Three CTL clones recognizing this peptide with high affinity were tested for lysis of PRAME and HLA-A*0201–expressing tumor cell lines at different E/T ratios (Table III). For comparison, three CTL clones recognizing the SLYSFPEPEA epitope (PRA$^{142–151}$) with equal affinity as the affinity of CTL anti-PRA$^{47–56}$ for LLPRELFPPL were included in the same experiment. The results show that CTLs raised against LLPRELFPPL do not lyse any of the tumor cell lines, whereas the same targets were efficiently killed by the control CTLs directed against SLYSFPEPEA (Table III). These data strongly suggest that LLPRELFPPL is not endogenously generated, supporting the accuracy of our epitope prediction procedure and the relevance of the proteasome digestion analysis. In addition to these data, high affinity CTL clones generated against three different BCR-ABL fusion protein–derived peptides failed to recognize BCR-ABL–expressing target cells (data not shown). Subsequent analysis of in vitro processing of these peptides showed that the proteasome did not generate the COOH terminus of any of these high affinity HLA class I binding peptides. These results confirm the importance of proper proteasomal cleavage for the generation of HLA class I–presented epitopes.

Table III. *Percent Specific Lysis of PRAME- and HLA-A*0201–Expressing Tumor Cell Lines by CTL Clones Specific for LLPRELFPPL (Not Processed) and SLYSFPEPEA (Processed) as Determined in a ^{51}Cr Cytotoxicity Assay*

		CTL clones anti-LLPRELFPPL			CTL clones anti-SLYSFPEPEA		
Tumor cell line*	E/T ratio	No. 3	No. 61	No. 120	No. 314	No. 343	No. 509
M453	6	2	2	10	33	39	26
	3	3	3	11	26	32	16
	1, 5	1	2	9	27	17	15
MZ1257	6	1	9	0	52	28	27
	3	1	8	4	34	16	26
	1, 5	2	8	2	32	12	12
MZ1774	6	7	8	10	43	42	31
	3	8	9	10	33	25	16
	1, 5	4	4	8	21	13	10
GLC36	6	2	10	7	26	28	18
	3	7	11	9	30	22	17
	1, 5	5	9	7	21	20	16
MCF7	6	5	8	7	56	26	32
	3	6	9	7	52	24	19
	1, 5	4	9	5	48	21	13
CTL sensitivity‡		0.1–1	0.1–1	0.1–1	0.1–1	0.1–1	0.1–1

*Cell lines used as targets are derived from melanoma (M453), renal cell cancer (MZ1257 and MZ1774), lung cancer (GLC36), and mammary cancer (MCF7).
‡CTL sensitivity for peptide expressed as [peptide] in nM at which the indicated CTL clone half-maximally lysed T2 cells pulsed with titrated amounts of peptide.

Discussion

In a systematic search for new CTL epitopes in known protein sequences with tumor restricted expression, the strategy of in vitro stimulation of CTLs with predicted epitopes (also coined "reverse immunology") has successfully led to the identification of several epitopes (18–25), but has met with many failures and is generally inefficient (our unpublished results and references 26–30). The current study reports the identification of four novel HLA-A*0201–restricted CTL epitopes in PRAME ($PRA^{100–108}$, $PRA^{142–151}$, $PRA^{300–309}$, and $PRA^{425–433}$) by an improved multistep epitope prediction procedure. Using in vitro proteasome-mediated digestion pattern analysis, the four epitopes were chosen for CTL inductions and shown to be naturally presented. In addition, we show that CTL clones with high sensitivity for high affinity binding peptide $PRA^{47–56}$, which was not produced in vitro by proteasome-mediated digestion (Fig. 2), were unable to lyse PRAME and HLA-A*0201–expressing tumor cell lines (Table III), indicating a lack of endogenous processing of this peptide. Taken together, both findings imply the accuracy and relevance of the proteasome-mediated digestion pattern analysis.

Importantly, only 4 out of the 19 peptides ($PRA^{100–108}$, $PRA^{142–151}$, $PRA^{300–309}$, and $PRA^{425–433}$) were COOH-terminally excised by a major cleavage site at 1 h incubation and were contained intact in digestion fragments as well, indicating possible abundantly expressed CTL epitopes. This reduction to 21% of high affinity HLA-A*0201 binding peptides being efficiently processed, which is in concordance with an estimation by Yewdell et al. (66), permitted us to avoid laborious and time consuming CTL inductions against unlikely epitopes. Indeed, the four predicted epitopes were all shown to be endogenously processed and presented (Figs. 4–6). For future epitope predictions in other proteins, it will be worthwhile to first systematically characterize proteasome digestion patterns of a complete set of overlapping long (e.g., 30-mer) polypeptides and subsequently determine binding affinities for MHC class I molecules of interest of only those peptides that are shown to be COOH-terminally excised by a major cleavage site. This experimental order reflects the physiological sequence of events, with the primacy of CTL epitope generation at antigen processing and not at MHC binding as indeed has been observed for MHC II epitope presentation (67).

Possibly, in the future, reliable proteasome cleavage prediction algorithms will allow by-passing of experimental digestions. Efforts to develop such algorithms have been reported (68, 69). By prediction algorithms of Kuttler et al. (69), three of the four epitopes identified in our study ($PRA^{142–151}$, $PRA^{300–309}$, and $PRA^{425–433}$) were predicted to be correctly COOH-terminally liberated. In contrast, the COOH terminus of $PRA^{100–108}$, which is generated by a major cleavage site in our study, was not predicted. Of the 25 major cleavage sites observed (Fig. 2), 17 sites (68%) were correctly predicted by the most optimal algorithm variant (type II) as well as 10 of the 23 (43%) minor cleavage sites observed at 1 h digestion (by algorithm variant type III). Furthermore, many cleavage sites were falsely predicted by the algorithms, including erroneous internal epitope destruction sites, thus reducing the value of the current algorithms. Although these differences may be partly attributable to the different types of proteasomes used (immunoproteasomes in this study versus constitutive proteasomes in reference 69), we conclude that this first accessible proteasome cleavage prediction algorithm is not yet accurate enough to be used without experimental verification. As suggested by Kuttler et al. (69), a further enlargement of the training data used to educate the algorithm is likely to improve the accuracy of prediction.

With respect to the utilization and interpretation of proteasome-mediated digestion patterns for epitope prediction as reported here, several points must be raised. For proteasome-mediated digestions, we used 20S proteasomes isolated from an EBV-transformed B cell line known to contain mainly so-called immunoproteasomes with LMP2, LMP7, and MECL1 subunits (58). This implies that the four identified epitopes are likely to be presented on full-length PRAME expressing mature DCs containing immunoproteasomes next to their expression on tumor cells (containing constitutive proteasomes). For whole antigen vaccine development, such epitopes are favorable in contrast to a type of CTL epitope of which the presentation has been reported to be abrogated in mature DCs (70). The reported reverse effect, an inefficient processing in cells containing constitutive proteasomes (71–74), is excluded for our CTL epitopes by the functional data (Figs. 4–6).

The selection of candidate epitopes was mainly based on generation of the correct COOH terminus by an early major cleavage site, which is considered a sine qua none for efficient epitope generation (34–37). Late emergence (at 4 or 24 h) of the correct COOH terminus or generation by a minor cleavage site is not expected to yield epitopes or only at very low density, respectively. In contrast, cleavage sites within the epitope were less heavily weighed in the epitope prediction. In particular, ALYVDSLFFL ($PRA^{300–309}$) was found to be cleaved at several sites within the epitope (Fig. 1 C). Although this phenomenon does not exclude epitope formation, as reported for epitopes of murine leukemia virus and CMV (37, 75), partial destruction of an epitope can severely hamper its efficient presentation (for a review by Niedermann et al., see reference 76). Despite that, the $PRA^{300–309}$ peptide was included in our CTL inductions for several reasons: (a) generation of its COOH terminus by a major cleavage site (Fig. 1 C), (b) reported presentation of the 9-mer length variant $PRA^{301–309}$ (LYVDSLFFL) in HLA-A24 (48), and (c) favorable binding capacity (Table I). Furthermore, (d) it can not be excluded that in vitro digestions are prone to a "recharging-effect": longer epitope precursor fragments (containing the correct COOH terminus) can be further degraded by reentry in the proteasome, a phenomenon which will presumably not occur in vivo, because translocation of polypeptides by

TAP to the ER has been shown to occur as soon as 15 min after protein synthesis (and consequently proteasome digestion [77]). However, our control experiments did not reveal a significant effect of peptide substrate concentration on the relative digestion fragment kinetics (data not shown), making pronounced effects of recharging unlikely and indicating that the observed kinetics at least partially reflect the primary cleavage pattern and fragment abundancy. Finally, (e) cleavage within this epitope may be diminished when digested with constitutive proteasomes instead of immunoproteasomes, as enhanced cleavages after leucine and valine (hydrophobic residues) by the latter type of proteasomes have been described (76).

Our data support the current notion that a significant proportion of CTL epitopes is produced by the proteasome as NH_2-terminally extended precursor (36, 38–41). Digestion analysis of PRAME 415–441 revealed that 9-mer $PRA^{425–433}$ is abundantly generated as NH_2-terminally elongated 10-mer $_{424}$QSLLQHLIGL$_{433}$ (Fig. 1 D), rendering it likely that this fragment is translocated by TAP to the ER and is trimmed there to its final length. Likewise, 9-mer $PRA^{100–108}$ is presumably formed as 11-mer $_{98}$KAVLDGLDVLL$_{108}$, which also indicates that the intermediate binding 10-mer $PRA^{99–108}$ may be presented as well (Fig. 1 A). In contrast, SLYSFPEPEA ($PRA^{142–151}$) is likely available for TAP translocation in its precise length, because this peptide was found in significant quantities as digestion fragment at 1 h digestion (Fig. 1 B). This is in concordance with a recent report showing that the proteasome can generate both COOH and NH_2 termini of some epitopes (78).

PRAME is a particularly attractive tumor-associated antigen because it is widely expressed in many different tumor types (48–51), but not in normal tissues, except testis, and at very low levels in endometrium, ovaries, and adrenals (48). Indeed, CTL clones recognizing the four novel HLA-A*0201–restricted PRAME epitopes specifically lysed melanoma, renal cell, lung, mammary, and cervical carcinoma cell lines (Figs. 4 and 6). Therefore, and given the high prevalence of HLA-A*0201 among the Caucasian population, these epitopes are expected to be applicable for immunotherapeutic purposes (adoptive CTL therapy, vaccine design, and/or immunomonitoring) in a high percentage of cancer patients. Undesirable autoimmune CTL reactivity against the few tissues expressing PRAME at low levels is not to be expected, because expression levels are most likely too low to ensure CTL recognition as shown in vitro with human MAGE-specific CTLs by Lethe et al. (79) and in vivo in a murine p53 model by our group (80). Nevertheless, control recognition studies with normal endometrium, ovary, or adrenal tissues should ascertain absence of harmful responses towards healthy tissues expressing PRAME at low levels (expression level $<$3–5% of that found in melanoma, with the exception of endometrium, which expresses up to 30% of the melanoma level [48]). So far we have been unable to establish sufficient primary cell cultures of those sources for functional analysis.

Although not the principle objective of this study, we found a remarkable immunogenicity in healthy donors of the four epitopes, because CTL inductions against the four peptides (performed with blood of two separate donors, see Materials and Methods) were all successful. Particularly, $PRA^{142–151}$ and $PRA^{425–433}$ vigorously induced CTL bulk cultures recognizing these peptides as endogenously expressed PRAME epitopes. Apparently, the low level expression of PRAME in some healthy tissues did not induce irreversible tolerance against the four identified epitopes. Future comparison of CTL frequencies in healthy donors versus cancer patients, as determined by, e.g., tetramer studies or enzyme-linked immunospot analysis, will reveal whether cancer patients are naturally primed against the four epitopes. Furthermore, such experiments may allow an immunodominance ranking of the epitopes. Our digestion data suggest a ranking in efficiencies of proteasome-mediated generation of the four epitopes, which is a major factor determining immunodominance (66, 76). Because of the higher abundance of epitope precursor fragments and absence of major cleavage sites within the epitopes, $PRA^{142–151}$ and $PRA^{425–433}$ are probably more efficiently generated than $PRA^{100–108}$ and $PRA^{300–309}$ (Figs. 1 and 2).

Finally, we expect that our novel epitope prediction methodology will help to rapidly identify PRAME-derived CTL epitopes presented in HLA class I molecules other than HLA-A*0201 and will boost the reverse immunology approach for other tumor specific proteins as well. Such a systematic identification of new CTL epitopes in different tumor antigens will allow the development of multiantigen (epitope-based) tumor vaccines, covering all HLA class I haplotypes, which is probably needed to circumvent tumor escape by antigen loss variants.

We thank Dr. P. Coulie for the gift of the PRAME cDNA and Mrs. W. Benckhuijsen for synthesis of peptides.

Submitted: 24 July 2000
Revised: 18 October 2000
Accepted: 30 October 2000

References

1. Melief, C.J.M., R.E. Toes, J.P. Medema, S.H. van der Burg, F. Ossendorp, and R. Offringa. 2000. Strategies for immunotherapy of cancer. *Adv. Immunol.* 75:235–281.
2. Hsu, F.J., C. Benike, F. Fagnoni, T.M. Liles, D. Czerwinski, B. Taidi, E.G. Engleman, and R. Levy. 1996. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat. Med.* 2:52–58.
3. Nestle, F.O., S. Alijagic, M. Gilliet, Y. Sun, S. Grabbe, R. Dummer, G. Burg, and D. Schadendorf. 1998. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat. Med.* 4:328–332.
4. Marchand, M., N. van Baren, P. Weynants, V. Brichard, B. Dreno, M.H. Tessier, E. Rankin, G. Parmiani, F. Arienti, Y. Humblet, et al. 1999. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int. J. Cancer.* 80:219–230.

5. Thurner, B., I. Haendle, C. Roder, D. Dieckmann, P. Keikavoussi, H. Jonuleit, A. Bender, C. Maczek, D. Schreiner, P. von den Driesch, et al. 1999. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J. Exp. Med.* 190:1669–1678.
6. Kolb, H.J., and E. Holler. 1997. Adoptive immunotherapy with donor lymphocyte transfusions. *Curr. Opin. Oncol.* 9:139–145.
7. Offringa, R., S.H. van der Burg, F. Ossendorp, R.E. Toes, and C.J. Melief. 2000. Design and evaluation of antigen-specific vaccination strategies against cancer. *Curr. Opin. Immunol.* 12:576–582.
8. Rosenberg, S.A. 1999. A new era for cancer immunotherapy based on the genes that encode cancer antigens. *Immunity.* 10: 281–287.
9. Van den Eynde, B.J., and P. van der Bruggen. 1997. T cell defined tumor antigens. *Curr. Opin. Immunol.* 9:684–693.
10. van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B.J. Van den Eynde, A. Knuth, and T. Boon. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science.* 254:1643–1647.
11. Traversari, C., P. van der Bruggen, I.F. Luescher, C. Lurquin, P. Chomez, A. Van Pel, E. De Plaen, A. Amar-Costesec, and T. Boon. 1992. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J. Exp. Med.* 176:1453–1457.
12. Van den Eynde, B.J., O. Peeters, O. De Backer, B. Gaugler, S. Lucas, and T. Boon. 1995. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. *J. Exp. Med.* 182:689–698.
13. Boel, P., C. Wildmann, M.L. Sensi, R. Brasseur, J.C. Renauld, P. Coulie, T. Boon, and P. van der Bruggen. 1995. BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes. *Immunity.* 2:167–175.
14. Brichard, V., A. Van Pel, T. Wolfel, C. Wolfel, E. De Plaen, B. Lethe, P. Coulie, and T. Boon. 1993. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 178:489–495.
15. Coulie, P.G., V. Brichard, A. Van Pel, T. Wolfel, J. Schneider, C. Traversari, S. Mattei, E. De Plaen, C. Lurquin, and J.P. Szikora. 1994. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 180:35–42.
16. Kawakami, Y., S. Eliyahu, C.H. Delgado, P.F. Robbins, L. Rivoltini, S.L. Topalian, T. Miki, and S.A. Rosenberg. 1994. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc. Natl. Acad. Sci. USA.* 91:3515–3519.
17. Bakker, A.B., M.W. Schreurs, A.J. de Boer, Y. Kawakami, S.A. Rosenberg, G.J. Adema, and C.G. Figdor. 1994. Melanocyte lineage–specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. *J. Exp. Med.* 179:1005–1009.
18. Fujie, T., K. Tahara, F. Tanaka, M. Mori, K. Takesako, and T. Akiyoshi. 1999. A MAGE-1-encoded HLA-A24-binding synthetic peptide induces specific anti-tumor cytotoxic T lymphocytes. *Int. J. Cancer.* 80:169–172.
19. Kawashima, I., S.J. Hudson, V. Tsai, S. Southwood, K. Takesako, E. Appella, A. Sette, and E. Celis. 1998. The multi-epitope approach for immunotherapy for cancer: identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors. *Hum. Immunol.* 59:1–14.
20. Celis, E., V. Tsai, C. Crimi, R. DeMars, P.A. Wentworth, R.W. Chesnut, H.M. Grey, A. Sette, and H.M. Serra. 1994. Induction of anti-tumor cytotoxic T lymphocytes in normal humans using primary cultures and synthetic peptide epitopes. *Proc. Natl. Acad. Sci. USA.* 91:2105–2109.
21. van der Bruggen, P., J. Bastin, T. Gajewski, P.G. Coulie, P. Boel, C. De Smet, C. Traversari, A. Townsend, and T. Boon. 1994. A peptide encoded by human gene MAGE-3 and presented by HLA-A2 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE-3. *Eur. J. Immunol.* 24:3038–3043.
22. Herman, J., P. van der Bruggen, I.F. Luescher, S. Mandruzzato, P. Romero, J. Thonnard, K. Fleischhauer, T. Boon, and P.G. Coulie. 1996. A peptide encoded by the human MAGE3 gene and presented by HLA-B44 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE3. *Immunogenetics.* 43:377–383.
23. Tanaka, F., T. Fujie, K. Tahara, M. Mori, K. Takesako, A. Sette, E. Celis, and T. Akiyoshi. 1997. Induction of antitumor cytotoxic T lymphocytes with a MAGE-3-encoded synthetic peptide presented by human leukocytes antigen-A24. *Cancer Res.* 57:4465–4468.
24. Parkhurst, M.R., E.B. Fitzgerald, S. Southwood, A. Sette, S.A. Rosenberg, and Y. Kawakami. 1998. Identification of a shared HLA-A*0201-restricted T-cell epitope from the melanoma antigen tyrosinase-related protein 2 (TRP2). *Cancer Res.* 58:4895–4901.
25. Tsai, V., S. Southwood, J. Sidney, K. Sakaguchi, Y. Kawakami, E. Appella, A. Sette, and E. Celis. 1997. Identification of subdominant CTL epitopes of the GP100 melanoma-associated tumor antigen by primary in vitro immunization with peptide-pulsed dendritic cells. *J. Immunol.* 158: 1796–1802.
26. van Elsas, A., H.W. Nijman, C.E. van der Minne, J.S. Mourer, W.M. Kast, C.J. Melief, and P.I. Schrier. 1995. Induction and characterization of cytotoxic T-lymphocytes recognizing a mutated p21ras peptide presented by HLA-A*0201. *Int. J. Cancer.* 61:389–396.
27. Nijman, H.W., S.H. van der Burg, M.P. Vierboom, J.G. Houbiers, W.M. Kast, and C.J. Melief. 1994. p53, a potential target for tumor-directed T cells. *Immunol. Lett.* 40:171–178.
28. Gedde-Dahl, T., III, A. Spurkland, B. Fossum, A. Wittinghofer, E. Thorsby, and G. Gaudernack. 1994. T cell epitopes encompassing the mutational hot spot position 61 of p21 ras. Promiscuity in ras peptide binding to HLA. *Eur. J. Immunol.* 24:410–414.
29. Disis, M.L., J.W. Smith, A.E. Murphy, W. Chen, and M.A. Cheever. 1994. In vitro generation of human cytolytic T-cells specific for peptides derived from the HER-2/neu protooncogene protein. *Cancer Res.* 54:1071–1076.
30. Zaks, T.Z., and S.A. Rosenberg. 1998. Immunization with a peptide epitope (p369-377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER- 2/neu1 tumors. *Cancer Res.* 58:4902–4908.
31. Chaux, P., R. Luiten, N. Demotte, V. Vantomme, V. Stroobant, C. Traversari, V. Russo, E. Schultz, G.R. Cornelis, T. Boon, and B.P. van der Bruggen. 1999. Identification of five MAGE-A1 epitopes recognized by cytolytic T lym-

phocytes obtained by In vitro stimulation with dendritic cells transduced with MAGE-A1. *J. Immunol.* 163:2928–2936.
32. York, I.A., and K.L. Rock. 1996. Antigen processing and presentation by the class I major histocompatibility complex. *Annu. Rev. Immunol.* 14:369–396.
33. Pamer, E., and P. Cresswell. 1998. Mechanisms of MHC class I-restricted antigen processing. *Annu. Rev. Immunol.* 16: 323–358.
34. Craiu, A., T. Akopian, A. Goldberg, and K.L. Rock. 1997. Two distinct proteolytic processes in the generation of a major histocompatibility complex class I-presented peptide. *Proc. Natl. Acad. Sci. USA.* 94:10850–10855.
35. Snyder, H.L., I. Bacik, J.W. Yewdell, T.W. Behrens, and J.R. Bennink. 1998. Promiscuous liberation of MHC-class I-binding peptides from the C termini of membrane and soluble proteins in the secretory pathway. *Eur. J. Immunol.* 28: 1339–1346.
36. Mo, X.Y., P. Cascio, K. Lemerise, A.L. Goldberg, and K. Rock. 1999. Distinct proteolytic processes generate the C and N termini of MHC class I-binding peptides. *J. Immunol.* 163:5851–5859.
37. Beekman, N.J., P.A. van Veelen, T. van Hall, A. Neisig, A. Sijts, M. Camps, P.M. Kloetzel, J.J. Neefjes, C.J. Melief, and F. Ossendorp. 2000. Abrogation of CTL epitope processing by single amino acid substitution flanking the C-terminal proteasome cleavage site. *J. Immunol.* 164:1898–1905.
38. Snyder, H.L., J.W. Yewdell, and J.R. Bennink. 1994. Trimming of antigenic peptides in an early secretory compartment. *J. Exp. Med.* 180:2389–2394.
39. Roelse, J., M. Gromme, F. Momburg, G. Hammerling, and J. Neefjes. 1994. Trimming of TAP-translocated peptides in the endoplasmic reticulum and in the cytosol during recycling. *J. Exp. Med.* 180:1591–1597.
40. Elliott, T., A. Willis, V. Cerundolo, and A. Townsend. 1995. Processing of major histocompatibility class I-restricted antigens in the endoplasmic reticulum. *J. Exp. Med.* 181:1481–1491.
41. Hughes, E.A., B. Ortmann, M. Surman, and P. Cresswell. 1996. The protease inhibitor, *N*-acetyl-*L*-leucyl-*L*-leucyl-leucyl-*L*-norleucinal, decreases the pool of major histocompatibility complex class I–binding peptides and inhibits peptide trimming in the endoplasmic reticulum. *J. Exp. Med.* 183:1569–1578.
42. Beninga, J., K.L. Rock, and A.L. Goldberg. 1998. Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. *J. Biol. Chem.* 273:18734–18742.
43. Dick, L.R., C. Aldrich, S.C. Jameson, C.R. Moomaw, B.C. Pramanik, C.K. Doyle, G.N. Demartino, M.J. Bevan, J.M. Forman, and C.A. Slaughter. 1994. Proteolytic processing of ovalbumin and beta-galactosidase by the proteasome to a yield antigenic peptides. *J. Immunol.* 152:3884–3894.
44. Eggers, M., B. Boes-Fabian, T. Ruppert, P.M. Kloetzel, and U.H. Koszinowski. 1995. The cleavage preference of the proteasome governs the yield of antigenic peptides. *J. Exp. Med.* 182:1865–1870.
45. Niedermann, G., S. Butz, H.G. Ihlenfeldt, R. Grimm, M. Lucchiari, H. Hoschutzky, G. Jung, B. Maier, and K. Eichmann. 1995. Contribution of proteasome-mediated proteolysis to the hierarchy of epitopes presented by major histocompatibility complex class I molecules. *Immunity.* 2:289–299.
46. Niedermann, G., G. King, S. Butz, U. Birsner, R. Grimm, J. Shabanowitz, D.F. Hunt, and K. Eichmann. 1996. The proteolytic fragments generated by vertebrate proteasomes: structural relationships to major histocompatibility complex class I binding peptides. *Proc. Natl. Acad. Sci. USA.* 93:8572–8577.
47. Ossendorp, F., M. Eggers, A. Neisig, T. Ruppert, M. Groettrup, A. Sijts, E. Mengede, P.M. Kloetzel, J. Neefjes, U. Koszinowski, and C. Melief. 1996. A single residue exchange within a viral CTL epitope alters proteasome-mediated degradation resulting in lack of antigen presentation. *Immunity.* 5:115–124.
48. Ikeda, H., B. Lethe, F. Lehmann, N. van Baren, J.F. Baurain, C. De Smet, H. Chambost, M. Vitale, A. Moretta, T. Boon, and P.G. Coulie. 1997. Characterization of an antigen that is recognized on a melanoma showing partial HLA loss by CTL expressing an NK inhibitory receptor. *Immunity.* 6:199–208.
49. van Baren, N., H. Chambost, A. Ferrant, L. Michaux, H. Ikeda, I. Millard, D. Olive, T. Boon, and P.G. Coulie. 1998. PRAME, a gene encoding an antigen recognized on a human melanoma by cytolytic T cells, is expressed in acute leukaemia cells. *Br. J. Haematol.* 102:1376–1379.
50. Neumann, E., A. Engelsberg, J. Decker, S. Storkel, E. Jaeger, C. Huber, and B. Seliger. 1998. Heterogeneous expression of the tumor-associated antigens RAGE-1, PRAME, and glycoprotein 75 in human renal cell carcinoma: candidates for T-cell-based immunotherapies? *Cancer Res.* 58:4090–4095.
51. Pellat-Deceunynck, C., M.P. Mellerin, N. Labarriere, G. Jego, A. Moreau-Aubry, J.L. Harousseau, F. Jotereau, and R. Bataille. 2000. The cancer germ-line genes MAGE-1, MAGE-3 and PRAME are commonly expressed by human myeloma cells. *Eur. J. Immunol.* 30:803–809.
52. Imanishi, T., T. Akaza, A. Kimura, K. Tokunaga, and T. Gojobori. 1992. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. *In* HLA 1991, Proceedings of the Eleventh International Histocompatibility Workshop and Conference. K. Tjuji, M. Aizawa, and T. Sasazuki, editors. Oxford University Press, Oxford/New York/Tokyo. 1065–1220.
53. Garrone, P., E.M. Neidhardt, E. Garcia, L. Galibert, C. van Kooten, and J. Banchereau. 1995. Fas ligation induces apoptosis of CD40-activated human B lymphocytes. *J. Exp. Med.* 182:1265–1273.
54. van der Burg, S.H., E. Ras, J.W. Drijfhout, W.E. Benckhuijsen, A.J. Bremers, C.J. Melief, and W.M. Kast. 1995. An HLA class I peptide-binding assay based on competition for binding to class I molecules on intact human B cells. Identification of conserved HIV-1 polymerase peptides binding to HLA-A*0301. *Hum. Immunol.* 44:189–198.
55. Bertoletti, A., F.V. Chisari, A. Penna, S. Guilhot, L. Galati, G. Missale, P. Fowler, H.J. Schlicht, A. Vitiello, and R.C. Chesnut. 1993. Definition of a minimal optimal cytotoxic T-cell epitope within the hepatitis B virus nucleocapsid protein. *J. Virol.* 67:2376–2380.
56. van der Burg, S.H., M.J. Visseren, R.M. Brandt, W.M. Kast, and C.J. Melief. 1996. Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. *J. Immunol.* 156:3308–3314.
57. Groettrup, M., T. Ruppert, L. Kuehn, M. Seeger, S. Standera, U. Koszinowski, and P.M. Kloetzel. 1995. The interferon-inducible 11S regulator (PA28) and the LMP2/LMP7 subunits govern the peptide production by the 20S proteasome in vitro. *J. Biol. Chem.* 270:23808–23815.
58. Frisan, T., V. Levitsky, A. Polack, and M.G. Masucci. 1998. Phenotype-dependent differences in proteasome subunit

composition and cleavage specificity in B cell lines. *J. Immunol.* 160:3281–3289.
59. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony–stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med.* 179:1109–1118.
60. Schultze, J.L., S. Michalak, M.J. Seamon, G. Dranoff, K. Jung, J. Daley, J.C. Delgado, J.G. Gribben, and L.M. Nadler. 1997. CD40-activated human B cells: an alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy. *J. Clin. Invest.* 100:2757–2765.
61. Cella, M., D. Scheidegger, K. Palmer-Lehmann, P. Lane, A. Lanzavecchia, and G. Alber. 1996. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* 184:747–752.
62. D'Amaro, J., J.G. Houbiers, J.W. Drijfhout, R.M. Brandt, R. Schipper, J.N. Bavinck, C.J. Melief, and W.M. Kast. 1995. A computer program for predicting possible cytotoxic T lymphocyte epitopes based on HLA class I peptide-binding motifs. *Hum. Immunol.* 43:13–18.
63. Parker, K.C., M.A. Bednarek, and J.E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide sidechains. *J. Immunol.* 152:163–175.
64. Rammensee, H.G., J. Bachman, and S. Stevanovic. 1997. MHC Ligands and Peptide Motifs. Springer-Verlag, Heidelberg, Germany. 462 pp.
65. van de Corput, L., H.C. Kluin-Nelemans, M.G. Kester, R. Willemze, and J.H. Falkenburg. 1999. Hairy cell leukemia-specific recognition by multiple autologous HLA-DQ or DP-restricted T-cell clones. *Blood.* 93:251–259.
66. Yewdell, J.W., and J.R. Bennink. 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu. Rev. Immunol.* 17:51–88.
67. Phelps, R.G., V.L. Jones, M. Coughlan, A.N. Turner, and A.J. Rees. 1998. Presentation of the Goodpasture autoantigen to CD4 T cells is influenced more by processing constraints than by HLA class II peptide binding preferences. *J. Biol. Chem.* 273:11440–11447.
68. Holzhutter, H.G., C. Frommel, and P.M. Kloetzel. 1999. A theoretical approach towards the identification of cleavage-determining amino acid motifs of the 20 S proteasome. *J. Mol. Biol.* 286:1251–1265.
69. Kuttler, C., A.K. Nussbaum, T.P. Dick, H.G. Rammensee, H. Schild, and K.P. Hadeler. 2000. An algorithm for the prediction of proteasomal cleavages. *J. Mol. Biol.* 298:417–429. Algorithm accessible at: www.uni-tuebingen.de/uni/kxi/contents.html.
70. Morel, S., F. Levy, O. Burlet-Schiltz, F. Brasseur, M. Probst-Kepper, A.L. Peitrequin, B. Monsarrat, R. Van Velthoven, J.C. Cerottini, T. Boon, et al. 2000. Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells. *Immunity.* 12:107–117.
71. Schwarz, K., M. van den Broek, S. Kostka, R. Kraft, A. Soza, G. Schmidtke, P.M. Kloetzel, and M. Groettrup. 2000. Overexpression of the proteasome subunits LMP2, LMP7, and MECL-1, but not PA28alpha/beta, enhances the presentation of an immunodominant lymphocytic choriomeningitis virus T cell epitope. *J. Immunol.* 165:768–778.
72. Sijts, A.J., T. Ruppert, B. Rehermann, M. Schmidt, U. Koszinowski, and P.M. Kloetzel. 2000. Efficient generation of a hepatitis B virus cytotoxic T lymphocyte epitope requires the structural features of immunoproteasomes. *J. Exp. Med* 191: 503–514.
73. Sijts, A.J., S. Standera, R.E. Toes, T. Ruppert, N.J. Beekman, P.A. van Veelen, F.A. Ossendorp, C.J. Melief, and P.M. Kloetzel. 2000. MHC class I antigen processing of an adenovirus CTL epitope is linked to the levels of immunoproteasomes in infected cells. *J. Immunol.* 164:4500–4506.
74. van Hall, T., A. Sijts, M. Camps, R. Offringa, C. Melief, P.M. Kloetzel, and F. Ossendorp. 2000. Differential influence on cytotoxic T lymphocyte epitope presentation by controlled expression of either proteasome immunosubunits or PA28. *J. Exp. Med.* 192:483–494.
75. Boes, B., H. Hengel, T. Ruppert, G. Multhaup, U.H. Koszinowski, and P.M. Kloetzel. 1994. Interferon γ stimulation modulates the proteolytic activity and cleavage site preference of 20S mouse proteasomes. *J. Exp. Med.* 179:901–909.
76. Niedermann, G., E. Geier, M. Lucchiari-Hartz, N. Hitziger, A. Ramsperger, and K. Eichmann. 1999. The specificity of proteasomes: impact on MHC class I processing and presentation of antigens. *Immunol. Rev.* 172:29–48.
77. Reits, E.A., J.C. Vos, M. Gromme, and J. Neefjes. 2000. The major substrates for TAP in vivo are derived from newly synthesized proteins. *Nature.* 404:774–778.
78. Lucchiari-Hartz, M., P.M. van Endert, G. Lauvau, R. Maier, A. Meyerhans, D. Mann, K. Eichmann, and G. Niedermann. 2000. Cytotoxic T lymphocyte epitopes of HIV-1 Nef: generation of multiple definitive major histocompatibility complex class I ligands by proteasomes. *J. Exp. Med.* 191:239–252.
79. Lethe, B., P. van der Bruggen, F. Brasseur, and T. Boon. 1997. MAGE-1 expression threshold for the lysis of melanoma cell lines by a specific cytotoxic T lymphocyte. *Melanoma Res.* 7(Suppl. 2):S83–S88.
80. Vierboom, M.P., H.W. Nijman, R. Offringa, E.I. van der Voort, T. van Hall, L. van den Broek, G.J. Fleuren, P. Kenemans, W.M. Kast, and C.J. Melief. 1997. Tumor eradication by wild-type p53-specific cytotoxic T lymphocytes. *J. Exp. Med.* 186:695–704.