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, PRA190–196; SLYSPEPES, PRA242–248; ALYVDLFFL, PRA300–306; and SLQHLGL, PRA325–333) 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
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) 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 NH2-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).

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 FMS), 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 hygromycin resistance. The renal cell carcinoma cell line MZ1851 was transfected with pDR2-PRAME using Fugene (Boehringer) as transfection reagent. After 48 h, hygromycin (100 µg/ml) was added to select transfected cells. Hygromycin-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-fluorenylmethylxyloxy carbonyl (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 6HLPSDYFPSV (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: FLPSDYFPSV (54). The 27-mer polypeptides used for in vitro proteasome digestion were synthesized as described above, purified by reversed phase–HPLC in an acetoni trile–water gradient and lyophilized from acetoni trile–water overnight. Purity was confirmed by mass spectrometry.

Abbreviations used in this paper: aa, amino acid; B-LCL, B lymphoblastoid cell line; DC, dendritic cell; ER, endoplasmic reticulum; FL, fluorescein; HBV, hepatitis B virus; MS, mass spectrometry; RT, reverse transcription; TAP, transporter-associated with antigen processing.
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 Na2HPO4). 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 β2-microglobulin (Sigma-Aldrich). Subsequently, the stripped JY cells were plated at 4 × 10^5/well in a 96-well V-bottomed plate together with 150 nM of a known high affinity HLA-A*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 FACSscan™ flow cytometer (Becton Dickinson). The percentage inhibition of FL-labeled reference peptide binding was calculated by using the following formula: [1 - (MF fluorescence and competitor peptide - MF fluorescence reference peptide)/(MF fluorescence reference peptide - MF fluorescence reference peptide)] × 100%. The binding affinity of competitor peptide is expressed as the concentration needed to inhibit 50% binding of the FL-labeled reference peptide (IC50). An IC50 ≤ 5 μM was considered high affinity binding, 5 μM < IC50 ≤ 15 μM was considered intermediate affinity binding, 15 μM < IC50 ≤ 100 μM was judged low affinity binding, and IC50 > 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 digestion 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]). The 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/height 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 Biolyx/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 5X reverse transcriptase buffer (Promega), 2.5 μl each of 10 mM deoxynucleotides (Amersham Pharmacia Biotech), 0.5 μg oligo dT15 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′-CTG TACT- CATT TCCAGACGAGA-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 PRA90-99 and the other donor for induction against PRA100-109) and PRA54-63, and PRA57-66) were obtained by the Ficoll-Paque method and used for CTL in...
tide titration experiments, 51Cr-labeled target cells (2,000/well) pulsed with 50 ng/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 microwells 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 1% HPS, 5 ng/ml IL-7, and 0.1 ng/ml IL-12. Autologous-activated B cells, generated via the protocol described by Schulze 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 β2-microglobulin (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.

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 (IC50 ≤ 6 μM), and 27 peptides bound with intermediate affinity (6 μM < IC50 ≤ 15 μM), whereas the other peptides displayed a low (15 μM < IC50 ≤ 100 μM) or undetectable binding capacity (IC50 > 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 (PRA292–301 and PRA190–199) 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, PRA292–301 and PRA190–199 were, with respect to their binding characteristics, not likely to be efficiently presented in HLA-A*0201. For all other peptides, the 50% decay time (DT50) was 2.5 h or longer (Table II), indicating a stable association with HLA-A*0201.

In Vivo 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 | 9 | 1.9  | 33 | SLLKDEALAI | 10 | 14.0 | 240 | VCTCTKLPTL | 10 | 67.2 |
| 47     | LLLPRLFPPPL | 10 | 2.1  | 422 | ALQSLQHQL | 9 | 14.2 | 44 | ALELPREL | 9 | 71.3 |
| 435    | NLTQHLYPVP | 9 | 2.5  | 103 | GLDVLLAQEV | 10 | 15.2 | 379 | TLQLDVLFDEC | 10 | 71.9 |
| 292    | FLSLQCLQLAL | 10 | 2.5  | 231 | GLDSLEDIAE | 10 | 15.5 | 371 | ALLERASAT | 9 | 72.0 |
| 394    | QLLALLPSL | 9 | 2.9  | 312 | RLQDLLRHKV | 9 | 15.7 | 353 | VLSLSGVMLT | 9 | 74.6 |
| 182    | FLKEGACDEL | 10 | 3.0  | 493 | RTFYPDEPI | 9 | 15.8 | 305 | SLFFLRGLR | 9 | 79.4 |
| 294    | SLQCLQLYVL | 10 | 3.2  | 308 | FLRGLDQQL | 9 | 16.1 | 409 | TTLSFYGNIS | 10 | 80.8 |
| 422    | ALQLSLQHQLI | 9 | 3.2  | 429 | HILGSLTLH | 10 | 16.3 | 93 | HLETFKAVL | 9 | 89.0 |
| 425    | ASSLQHQLGL | 9 | 3.7  | 85 | VLMKQHQLHL | 10 | 17.0 | 319 | HVMNPLETL | 9 | 90.3 |
| 258    | QMNLRRLILL | 10 | 4.0  | 316 | LLRHSVNNPL | 9 | 17.4 | 18 | SVVTSRRLIV | 10 | 100 |
| 190    | ELFSYLIEKV | 10 | 4.5  | 353 | VLSLGSVML | 9 | 17.4 | 20 | WSFRRLVEL | 10 | 100 |
| 248    | TLAKFSYPYL | 9 | 4.6  | 172 | FIPQVKLDVL | 10 | 17.5 | 26 | VLSLQGQL | 9 | 100 |
| 39     | ALAIAAALEL | 9 | 5.1  | 134 | TVWSGNRASL | 10 | 18.4 | 51 | ELFPPFLMA | 9 | 100 |
| 100    | VLDDLVDVI | 9 | 5.2  | 339 | VHHLQSSPSV | 10 | 18.5 | 67 | QTLKAMQVA | 9 | 100 |
| 333    | RLSEGDVMHL | 9 | 5.4  | 72 | MVQWQFPTC | 9 | 18.5 | 70 | KAMVQAWFPT | 10 | 100 |
| 462    | YLHARREL | 9 | 5.4  | 390 | IDTDQDLLALL | 10 | 18.9 | 78 | PTCLPGVL | 9 | 100 |
| 360    | MLTDVSPEPL | 9 | 5.6  | 18 | SVWTSFPRRL | 10 | 19.1 | 84 | GVLKQGQHL | 9 | 100 |
| 419    | SISALQSL | 9 | 5.7  | 315 | QLLRRVNNPL | 10 | 19.7 | 95 | ETTFKAVLGD | 10 | 100 |
| 432    | GLSNLIYV | 10 | 6.7  | 81 | AMQVQAWFT | 9 | 20.0 | 133 | WTWVSQNR | 9 | 100 |
| 214    | KIFAMPQDI | 10 | 7.2  | 207 | RLQCKLKLI | 9 | 20.8 | 155 | MTKKRKVDGL | 10 | 100 |
| 320    | VMNPLETLSI | 10 | 8.6  | 247 | PLLAKFSYPY | 10 | 21.1 | 165 | STEAEQFPF | 9 | 100 |
| 39     | ALAIAAALEL | 9 | 9.0  | 219 | PVIQKIMKL | 9 | 23.9 | 180 | DLFLEKGCAG | 9 | 100 |
| 390    | ITDDQQLLL | 9 | 9.2  | 459 | RLAYLHAR | 9 | 24.3 | 198 | KVKRRKNVL | 9 | 100 |
| 242    | CTKWKLPTLA | 9 | 9.3  | 264 | RLLLSHIAA | 9 | 24.6 | 205 | VLRLLCKKVL | 9 | 100 |
| 99     | AVLGLDLVV | 10 | 9.4  | 217 | AMPQMKDIKM | 10 | 24.6 | 222 | DIKMLKHK | 9 | 100 |
| 308    | FLRGRDLQQL | 10 | 9.6  | 361 | LTVDVSPEPL | 9 | 26.8 | 224 | KMILKKVQ | 9 | 100 |
| 355    | SLSGVMFTLDV | 10 | 9.9  | 430 | LIGLSLTHLV | 10 | 27.2 | 234 | SIEDLEVTC | 9 | 100 |
| 34     | LLDDEALAI | 9 | 10.2 | 33 | SLLKDEALAI | 9 | 29.2 | 234 | SIEDLEVTC | 10 | 100 |
| 284    | YIAQFTSQFL | 10 | 10.4 | 258 | QMNLRRLLL | 9 | 31.2 | 237 | DLETVCTWLK | 10 | 100 |
| 784    | AMVQWQFPTC | 10 | 10.4 | 91 | HLHLETFKAV | 10 | 31.8 | 240 | VTTCTLKL | 9 | 100 |
| 470    | LLLCGLRPMS | 9 | 10.5 | 297 | CLQALYDSDL | 10 | 33.8 | 261 | NLRLLLSH1 | 9 | 100 |
| 186    | GACDELFPSY | 10 | 10.6 | 372 | LLERASATL | 9 | 35.0 | 325 | ETLSITNCRL | 10 | 100 |
| 410    | TLSFYGNIS | 9 | 11.0 | 401 | SLQHCSQGSLT | 9 | 36.9 | 368 | PLQALLERA | 9 | 100 |
| 25     | RVLWLAGQSL | 10 | 11.1 | 397 | ALPLSSSCH | 9 | 42.6 | 382 | DLVFDEGCQ | 9 | 100 |
| 91     | HLHLETFKAV | 9 | 11.1 | 389 | GITTDQQLLAL | 10 | 47.3 | 382 | DLVFDEGCQ | 10 | 100 |
| 100    | VLGLDVLVLA | 10 | 11.9 | 417 | SISIALQSL | 10 | 48.2 | 383 | LVSFDEGCQ | 9 | 100 |
| 454    | TLHLERAYL | 10 | 12.2 | 259 | MINLRLLL | 9 | 48.2 | 389 | GITTDQLLA | 9 | 100 |
| 371    | ALLERASATL | 10 | 12.9 | 479 | NVWLSANPC | 9 | 49.0 | 401 | SLSHCSQLTT | 10 | 100 |
| 326    | TLSTINCR | 9 | 13.2 | 160 | KVDGLSTEA | 9 | 51.2 | 473 | ELGRPSMVWL | 10 | 100 |
| 462    | YLHARLEL | 10 | 13.3 | 436 | LTVHYFVFL | 10 | 53.0 | 481 | WLSANFCFHC | 10 | 100 |
| 350    | QLSVLSLSGV | 10 | 13.3 | 226 | IKMVQDLLSI | 10 | 56.1 | 493 | RTFYDPFPEL | 10 | 100 |
| 99     | AVLGLDLVL | 9 | 13.4 | 292 | FLSLQCLQAA | 9 | 58.7 | 100 | |

*Position in PRAME of the NH₂-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 epitopes (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 VLGLDVYLL (PRA100–108) and the 10-mer AVLGLDVYLL (PRA99–108) represent potential CTL epitopes. The NH2 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 (PRA142–151), 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 IC50§ | Stability DT50¶ |
|--------|-----------|----------------|---------------|
| 300    | ALYVDSLFFL| 1.7            | >4            |
| 142    | SLYSFPEPEA| 1.9            | 3             |
| 47     | LLPRELFPFL| 2.1            | 2.5           |
| 435    | NLTHVLVPYV| 2.5            | 3             |
| 292    | FLSLQCLQAL| 2.5            | N.S.§         |
| 394    | QLLALLPSL | 2.9            | >4            |
| 182    | FLKEGACDEL| 3.0            | 3             |
| 294    | SLQLCLQALV| 3.2            | >4            |
| 422    | ALQSSLQHLI| 3.2            | 2.5           |
| 425    | SLLLHLGLL| 3.7            | >4            |
| 258    | QMNLRRLLL| 4.0            | >4            |
| 190    | ELFSYLIEXV| 4.5            | N.S. §        |
| 248    | TLAKFSPYLL| 4.6            | >4            |
| 100    | VLGLDVYLL| 5.2            | 2.5           |

*Start aa position of peptide in PRAME.
†In addition to all high affinity binding peptides, PRAME 100–108 and 371–380 are tested.
‡Affinity expressed as ICIC50 (see Table I).
¶DT50 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 (PRA290–307), 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 (PRA291–309; 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, PRA290–309 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 PRA292–301 and PRA294–303 (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 NH2-terminally elongated decameric precursor 43QSLLQHLIGL433 of high affinity binding 9-mer SLLQHLIGL (PRA425–433) was efficiently generated. The abundant generation of the COOH-terminal and NH2-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 424QSLLQHLIGL433, indicating PRA425–433, a potential CTL epitope. The three other HLA-A*0201 binding peptides were either not COOH-terminally excised (PRA419–427 and PRA422–431) or the correct COOH terminus was found only after 4 h incubation (PRA422–431).

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 (PRA47–56, PRA435–443, PRA92–103, PRA182–191, PRA208–209, PRA100–108, and PRA360–369) or the correct COOH terminus was generated only after 4 h incubation by a minor cleavage site (PRA291–402, PRA222–431, PRA190–199, and PRA419–427). 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; PRA258–267, PRA333–342, and PRA462–470), whereas PRA294–303 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 PRA100–108, PRA142–151, PRA106–109, and PRA422–431 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 Vivo Human CTL Inductions against Four Putative HLA-A*0201–restricted Epitopes. Separate CTL inductions, using PBMCs of healthy donors, were performed against VLDGLDVYLL (PRA100–108), SLYSFPEPEA
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 incubated 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 digestion 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) IC50 as determined in competition binding assay (see Table I); (2) peptides binding with high or intermediate affinity to HLA-A*0201 are shown. Predicted epitope prediction are not dered according to their COOH terminus; (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 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.

A Digestion PRAME 90–116

| Fragment | Intensity (% digested) |
|----------|------------------------|
| 90–97    | 26.19.29               |
| 93–101   | 8.2.3                  |
| 90–102   | 5.2.2                  |
| 90–108   | 2.1.0                  |
| 96–108   | 2.1.3                  |
| 100–108  | 0.1.0.2                |
| 103–108  | 4.1.2                  |
| 93–116   | 7.31.18                |
| 98–114   | 29.28.27               |
| 102–116  | 8.5.6                  |
| 103–116  | 7.5.6                  |

B Digestion PRAME 133–159

| Fragment | Intensity (% digested) |
|----------|------------------------|
| 133–136  | 22.35.28               |
| 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                |

C Digestion PRAME 290–316

| Fragment | Intensity (% digested) |
|----------|------------------------|
| 290–295  | 40.45.32               |
| 295–297  | 4.4.1                  |
| 297–303  | 4.5.4                  |
| 298–304  | 5.5.4                  |
| 300–306  | 2.2.2                  |
| 299–306  | 4.3.4                  |
| 299–309  | 0.8.0.4                |
| 300–309  | 0.0.0.3                |
| 301–309  | 0.0.0.7                |
| 304–309  | 4.5.9                  |
| 305–309  | 8.9.24                 |
| 309–318  | 21.19.13               |
| 304–316  | 7.5.3                  |

D Digestion PRAME 415–441

| Fragment | Intensity (% digested) |
|----------|------------------------|
| 415–420  | 13.12.6                 |
| 415–427  | 17.15.14               |
| 421–427  | 0.2.1                  |
| 421–431  | 0.4.2                  |
| 424–431  | 0.8.3                  |
| 422–433  | 0.1.7                  |
| 424–431  | 17.10.8                |
| 425–433  | 0.1.2                  |
| 427–441  | 0.8.8                  |
| 424–441  | 0.3.3                  |
| 432–441  | 0.5.3                  |
| 434–441  | 54.39.42                |
| HLA-A*0201 binding peptide | Digestion of 27-mer polypeptide | C-terminus | Intact fragment | Candidate epitope |
|-----------------------------|---------------------------------|------------|----------------|------------------|
| Start | Sequence | IC50 | 27-mer | Sequence with major and minor cleavage sites at 1 h digestion | 1 h | 4 or 24 h | |
| 300 | ALYVDLSLFLL | 1.7 | 290-316 | SQFLSLCCLOQALYVDSLLFRLRGLDQL | ++ | + | + |
| 142 | SLYSFPEPEA | 1.9 | 133-159 | WTVWSGNRASLYSFPEPEAQPMTKKR | ++ | + | + |
| 47 | LLPRELPPFP | 2.1 | 37-63 | DEALIAIAALEELLPRELLPLFMAMA | - | - | - |
| 435 | NTHLVLVPY | 2.5 | 424-450 | QSLQLHGILGLNLTHVLYPVPLESYED | - | - | - |
| 292 | FLSQLQOAL | 2.5 | 290-316 | SQFLSLCCLOQALYVDSLLFRLRGLDQL | - | - | - |
| 394 | QLLALLPDSL | 2.9 | 384-410 | VFDCEGIDQALLALLPDSLHCSQLTT | - | + | + |
| 182 | FLKGEACDEL | 3.0 | 173-199 | IPVEVLVLDLFLKGEACDELFSYLIEKV | - | - | - |
| 294 | SLOQLAYQ | 3.2 | 290-316 | SQFLSLCCLOQALYVDSLLFRLRGLDQL | + | + | + |
| 422 | AQLSHLOHL | 3.2 | 415-441 | GNSISIALQSLQLHLGLSNLTHVL | - | - | - |
| 425 | SLOQHLIG | 3.7 | 415-441 | GNSISIALQSLQLHLGLSNLTHVL | ++ | + | + |
| 258 | QMINLRLLL | 4.0 | 247-273 | PTLAKFSPYLOGMINLRLLLHSHIAS | + | + | + |
| 190 | ELFSLVEKV | 4.5 | 182-208 | FLSQACDELFSYLIEKVKRKNVLR | - | + | + |
| 248 | TLAKSFSPY | 4.6 | 239-265 | EVTCTWKLPTLAKFSPYLOGMINLRLLLHSHIAS | - | - | - |
| 39 | ALAIAAEL | 5.1 | 37-63 | DEALIAIAALEELLPRELPPFLMAMAFDG | - | - | - |
| 100 | VLGLDVLL | 5.2 | 90-116 | QHLHLETFKAVLGLDVLLAQEVPRPR | ++ | + | + |
| 333 | RLSEDQVMHL | 5.4 | 323-349 | PLETLSITNCRLSELDVMHLQGSPSVS | + | + | + |
| 460 | YLHARLREL | 5.6 | 460-486 | LAYLHARLRELCEGLRPSMVWLSANP | + | + | + |
| 360 | MLTDVSEPLE | 5.6 | 359-385 | VMELTDVSEPLEQALLERASATLQDLVF | - | - | - |
| 419 | SISALQGILL | 5.7 | 415-441 | GNSISIALQSLQLHLGLSNLTHVL | - | - | - |

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 at 1 h digestion are depicted. Notes: (1) all 19 high affinity binding peptides (IC50 < 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) IC50 (in μM) as determined in competition binding assay (see Table I); (4) C-terminus and N-terminus are shown for each peptide; (5) all 27-mer peptides encompassing the high affinity binding peptide; (6) major (bold arrows) and minor (thin arrows) cleavage sites at 1 h digestion are depicted, classified according to the following definitions. Major site: fragments containing as COOH terminus the residue NH2-terminal from the cleavage are present for ≥5% at 1 h incubation. Minor site: fragments containing as COOH terminus the residue NH2-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 NH2-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 VLGLDVLL (PRA100-108) showed specific lysis of peptide pulsed targets and 19 of 202 (9%) CTL clones raised against ALYVDLSLFL (PRA200-207) displayed peptide specificity. As may be expected, higher percentages, namely 92% (279 of 304), of clones against SLYSFPEPEA (PRA142-151) and 29% (97 of 336) of clones against SLOQHLIGL (PRA233-243) 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-PRA100-108) was able to half-maximally lyse targets loaded with VLGLDV-
VLL at ~5 nM peptide. CTL no. 314, raised against SLYS-FPEPEA (PRA\textsuperscript{142–151}), lysed T2 cells at half-maximal level when pulsed with <1 nM of the inducing peptide. CTL no. 460 (anti-PRA\textsuperscript{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\textsuperscript{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 TCR\textsubscript{B} transcripts from 22 well-established TCRBV families to determine V\textsubscript{B} usage of the TCR (65). All CTL clones were shown to use a single V\textsubscript{B}, confirming clonality of the clones (data not shown).

Figure 3. HLA-A\textsuperscript{0201}–restricted peptide specificity and sensitivity of CTL clones raised against four PRAME peptides. (A) Lysis by CTL clones no. 551 anti-PRA\textsuperscript{100–108}, no. 314 anti-PRA\textsuperscript{142–151}, no. 460 anti-PRA\textsuperscript{300–309}, and no. 1257 anti-PRA\textsuperscript{425–433} of \textsuperscript{51}Cr-labeled T2 cells loaded with 5 \textmu M of the relevant peptide (•) vs. an irrelevant HLA-A\textsuperscript{0201} binding peptide (○) at different E/T ratios ranging from 50 to 0.75. (B) Lysis by the same set of CTL clones of \textsuperscript{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.

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 \textsuperscript{51}Cr-labeled melanoma cell line Mel603, expressing PRAME but lacking HLA-A\textsuperscript{0201} expression (○), was tested vs. lysis of M453 (■) and FM3 (▲), both expressing PRAME and HLA-A\textsuperscript{0201} together. CTL clones no. 551 anti-PRA\textsuperscript{100–108}, no. 314 anti-PRA\textsuperscript{142–151}, no. 460 anti-PRA\textsuperscript{300–309}, and no. 1257 anti-PRA\textsuperscript{425–433} were used at E/T ratios ranging from 25 to 0.75. (Middle) Lysis of \textsuperscript{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 \textsuperscript{51}Cr-labeled FM3 was tested after 1 h treatment with 10 \textmu M of the proteasome inhibitor lactacystin (black bars) or without treatment (gray bar). 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.
Endogenous presentation of the candidate epitopes an HLA I–restricted and Proteasome-dependent Fashion. anoma Cell Lines Coexpressing HLA-A

MZ1851 transfected with the empty vector (H17033) was tested on 51Cr-labeled MZ1851 (HLA-A*0201* but lacking PRAME expression) transfected with PRAME cDNA (Δ) 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.

Figure 5. Lysis of PRAME transfected renal cell carcinoma cell line MZ1851 by CTL anti-PRA300–309. CTL no. 460 directed against PRAME (determined by Northern blotting, data not shown) but lacking HLA-A*0201 neg-tive (●) was compared. (Middle) Lysis of 51Cr-labeled renal cell carcinoma 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 51Cr-labeled lung carcinoma cell lines. GLC02, expressing PRAME but lacking HLA-A*0201 negative (●) and GLC36 (Δ) expressing both PRAME and HLA-A*0201 was compared. (Bottom) 51Cr-labeled mammary carcinoma cell lines MCF7 (HLA-A*0201* and PRAME*) (●) and ZR-75-1, expressing PRAME but lacking HLA-A*0201 (Δ), were tested. The CTL clones no. 551 anti-PRA100–108, no. 314 anti-PRA142–151, no. 460 anti-PRA300–309, and no. 1257 anti-PRA425–433 were used at E/T ratios ranging from 25 to 0.75. Results of one representative experiment of at least three performed are shown.

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 PRA100–108, PRA142–151, PRA300–309, and PRA425–433 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 51Cr 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-A*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 PRA425–433.

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 PRA100–108, PRA142–151, PRA300–309, and PRA425–433 on tumor cells from histologic origins other than melanoma, we used panels of cell lines derived from various tu-
morf 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 PRA100–108, PRA142–151, PRA300–309, and PRA425–433 are presented by HLA-A201–expressing tumor cell lines and PRAME+ tumor cell lines (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 51Cr Cytotoxicity Assay**

| 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                                       |
| MZ1257          | 6 1 9 0 52 28 27                                       |
|                 | 3 1 8 4 34 16 26                                       |
|                 | 1 5 2 8 32 12 12                                       |
| MZ1774          | 6 7 8 10 43 42 31                                       |
|                 | 3 8 9 10 33 25 16                                       |
|                 | 1 5 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 48 21 13                                       |

*Cell lines used as targets are derived from melanoma (M453), renal cell cancer (MZ1257 and MZ1774), lung cancer (GLC36), and mammary cancer (MCF7).

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.
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 (PRA100–108, PRA142–151, PRA300–309, and PRA425–433) 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 PRA47–56, 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 (PRA100–108, PRA142–151, PRA300–309, and PRA425–433) 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 (PRA142–151, PRA300–309, and PRA425–433) were predicted to be correctly COOH-terminally liberated. In contrast, the COOH terminus of PRA100–108, 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.

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 weighted in the epitope prediction. In particular, ALYVDSLFFL (PRA400–407) 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 PRA300–309 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 PRA301–309 (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

74 | Chapter 2 – CTL epitope identification in PRAME by improved prediction
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 abundance. Finally, (c) 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 NH2-terminally extended precursor (36, 38–41). Digestion analysis of PRAME 415–441 revealed that 9-mer PRA425–433 is abundantly generated as NH2-terminally elongated 10-mer R424QSLLQHLIGL433 (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 PRA100–108 is presumably formed as 11-mer KAVLDGLDVLL108, which also indicates that the intermediate binding 10-mer PRA99–108 may be presented as well (Fig. 1 A). In contrast, SLYSFPEPEA (PRA142–151) 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 NH2 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, PRA142–151 and PRA425–433 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, PRA142–151 and PRA425–433 are probably more efficiently generated than PRA100–108 and PRA96–100 (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. Benchkouwen 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. Osendorp, 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. Aljagic, M. Gillet, 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 recognition of tumor antigens. Curr. Opin. Immunol. 9:684–693.

10. van der Bruggen, P., C. Traversari, P. Chomez, C. Lerugn, E. De Plaen, B.J. Van den Eynde, A. Knuth, and T. Boon. 91. 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-Costescu, 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. Renaudil, P. Coulihe, T. Boon, and P. van der Bruggen. 1995. BAGE: a new gene encoding an antigen recognized on human melanomas by cytotoxic T lymphocytes. Immunity. 2:167–175.

14. Brichard, V., A. Van Pel, T. Wolfél, C. Wolfél, E. De Plaen, B. Lethé, P. Coulihe, and T. Boon. 1993. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanosomas. J. Exp. Med. 178:489–495.

15. Coulihe, P.G., V. Brichard, A. Van Pel, T. Wolfél, J. Schneider, C. Traversari, S. Mattei, E. De Plaen, C. Lerugn, and J.P. Szikora. 1994. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanosomas. J. Exp. Med. 180:35–42.

16. Kawakami, Y., S. Elyahu, 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. de Boer, Y. Kawakami, S.A. Rosenberg, G.J. Adema, and C.C. Fidler. 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. Cels. 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. Coulihe, 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. Coulihe. 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, C. 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. Cels. 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.J. 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. Dusi, 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/neu tumors. Cancer Res. 58:4902–4908.

31. Chaux, P., R. Luiten, N. Demotte, V. Vantomme, V. Stroobant, C. Traversari, V. Russo, E. Schulz, G.R. Cornelis, T. Boon, and B.P. van der Bruggen. 1999. Identification of five MAGE-A1 epitopes recognized by cytolytic T lymphocytes.
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. Paner, E., and P. Cresswell. 1998. Mechanisms of MHC class I-restricted antigen processing. Annu. Rev. Immunol. 16:323–358.
34. Crain, 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 terminus 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 Velen, T. van Hall, A. Neisig, A. Sijs, M. Camps, P.M. Kloeetzl, J.J. Neefjes, C.J. Melief, and F. Osendorp. 2000. Abrogation of CTL epitope processing by single amino acid substitution flanking the C-terminal proteasome cleavage site. J. Immunol. 164:1988–19905.
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. Roehe, J., M. Gromme, F. Mombarg, 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-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. Eukaryot. Cell 3:289–299.
44. Eggers, M., B. Boes-Fabian, T. Ruppert, P.M. Kloeetzl, 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. Lucchiarri, H. Hochstrzyk, 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. Birner, 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. Osendorp, F., M. Eggers, A. Neisig, T. Ruppert, M. Groettrup, P. Sijs, E. Mengelke, P.M. Kloeetzl, 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. Coulié. 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. Coulié. 1998. PRAME, a gene encoding an antigen recognized on a human melanoma by cytolytic T cells, is expressed in acute leukemia 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. Benchhuisen, 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. Virology. 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. Stand- era, U. Koszinowski, and P.M. Kloeetzl. 1995. The interferon-inducible 115k 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.

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.

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.

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.

D’Amaro, J., J.G. Houbiers, J.W. Drijfhout, R.M. Brandt, R. Schipper, J.N. Bavink, 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.

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 side-chains. J. Immunol. 152:163–175.

Rammensee, H.G., J. Bachman, and S. Stevanovic. 1997. MHC Ligands and Peptide Motifs. Springer-Verlag, Heidelberg, Germany. 462 pp.

van de Corput, L., H.C. Kluin-Nelemans, M.G. Kester, R. Rammensee, H.G., J. Bachman, and S. Stevanovic. 1997. Specific recognition by multiple autologous HLA-DQ or DQ-restricted T-cell clones. Blood. 93:251–259.

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.

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.

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.

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/content.html.

Morel, S., F. Levy, O. Burlet-Schultz, 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.

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 PA28α/β, enhances the presentation of an immunodominant lymphocytic choriomeningitis virus T cell epitope. J. Immunol. 165:768–778.

Sijs, 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.

Sijs, A.J., S. Stander, R.E. Toes, T. Ruppert, N.J. Beek, P.A. van Veelen, F.A. Osendorp, 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.

van Hall, T., A. Sijs, M. Camps, R. Offerings, C. Melief, P.M. Kloetzel, and F. Osendorp. 2000. Differential influence on cytotoxic T lymphocyte epitope presentation by controlled expression of either proteasome immunosubunits or PA28. J. Exp. Med. 192:483–494.

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.

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.

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.

Lucchiari-Hartz, M., P.M. van Endert, G. Lauvau, R. Maier, A. Meyerhas, 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.

Lethé, B., P. van der Bruggen, F. Brasseur, and T. Boon. 1997. MAGE-1 expression thresholds for the lysis of melanoma cell lines by a specific cytotoxic T lymphocyte. Melanoma Res. 7(Suppl. 2):S83–S88.

Vierboom, M.P., H.W. Nijman, R. Offerings, E.J. van der Voort, T. van Hall, L. van den Broek, G.J. Fleuren, P. Kenebrids, W.M. Kast, and C.J. Melief. 1997. Tumor eradication by wild-type p53-specific cytotoxic T lymphocytes. J. Exp. Med. 186:695–704.