A Spliced Antigenic Peptide Comprising a Single Spliced Amino Acid Is Produced in the Proteasome by Reverse Splicing of a Longer Peptide Fragment followed by Trimming

Alexandre Michaux, Pierre Larrieu, Vincent Stroobant, Jean-François Fonteneau, Francine Jotereau, Benoît J. Van den Eynde, Agnès Moreau-Aubry and Nathalie Vigneron

*J Immunol* 2014; 192:1962-1971; Prepublished online 22 January 2014;
doi: 10.4049/jimmunol.1302032
http://www.jimmunol.org/content/192/4/1962

---

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/01/22/jimmunol.1302032.DCSupplemental

**References**
This article cites 44 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/192/4/1962.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
A Spliced Antigenic Peptide Comprising a Single Spliced Amino Acid Is Produced in the Proteasome by Reverse Splicing of a Longer Peptide Fragment followed by Trimming

Alexandre Michaux, Pierre Larrieu, Vincent Stroobant, Jean-François Fonteneau, Francine Jotereau, Benoît J. Van den Eynde, Agnès Moreau-Aubry, and Nathalie Vigneron

Peptide splicing is a novel mechanism of production of peptides relying on the proteasome and involving the linkage of fragments originally distant in the parental protein. Peptides produced by splicing can be presented on class I molecules of the MHC and recognized by CTLs. In this study, we describe a new antigenic peptide, which is presented by HLA-A3 and comprises two non-contiguous fragments of the melanoma differentiation Ag gp100<sup>PMEL17</sup> spliced together in the reverse order to that in which they appear in the parental protein. Contrary to the previously described spliced peptides, which are produced by the association of fragments of 3–6 aa, the peptide described in this work results from the ultimate association of an 8-aa fragment with a single arginine residue. As described before, peptide splicing takes place in the proteasome by transpeptidation involving an acyl-enzyme intermediate linking one of the peptide fragment to a catalytic subunit of the proteasome. Interestingly, we observe that the peptide causing the nucleophilic attack on the acyl-enzyme intermediate must be at least 3 aa long to give rise to a spliced peptide. The spliced peptide produced from this reaction therefore bears an extended C terminus that needs to be further trimmed to produce the final antigenic peptide. We show that the proteasome is able to perform the final trimming step required to produce the antigenic peptide described in this work. The Journal of Immunology, 2014, 192: 1962–1971.

Cytotoxic T lymphocytes are major players of the antitumor response, as they are involved in the detection and clearance of tumor cells. These CTL generally recognize peptides of 8–10 aa that are derived from the degradation of cellular proteins and are presented at the cell surface by class I molecules of the MHC. A number of antitumor CTL were successfully expanded in vitro by culturing blood or tumor-infiltrating lymphocytes with autologous tumor cells or dendritic cells loaded with peptide or full-length proteins [reviewed in (1)]. Peptides recognized by these CTL were classified in four categories according to the expression profile of their parental gene (2, 3) (http://www.cancerimmunity.org/peptide/). The cancer germline genes, which include the melanoma Ag gene family, are expressed in a large variety of tumors but not in normal tissues, except the male germline cells (4, 5). Differentiation Ags such as tyrosinase, gp100<sup>PMEL17</sup>, or Melan-A/MART-1 are derived from genes displaying a tissue-restricted expression and are therefore expressed in corresponding tumors (6–11). Peptides also arise from point mutations (12–14) or genes that are overexpressed in tumors (15, 16).

Whereas most antigenic peptides result from the degradation of intracellular proteins by the proteasome, a number of peptides were described whose sequence does not correspond to that predicted from the parental gene or protein. Such peptides were found to arise from pseudogenes (17), from aberrant transcription of intronic or reverse-strand sequences (18, 19), and from the translation of alternative open-reading frames (20) or posttranslational modifications such as threonine/serine phosphorylation (21) or asparagine deamination (22, 23) [reviewed in (2)]. More recently, we and others described peptides composed of two noncontiguous fragments of a protein that are bound together after the excision of their intervening segment. This process, termed peptide splicing, takes place as a side effect of proteolysis inside the catalytic chamber of the proteasome. Four spliced peptides were described to date. A first peptide, identified by Hanada et al. (24), is derived from the fibroblast growth factor-5 (FGF-5) and presented by the HLA-A3. It originates from the splicing of fragments NTYA and PRFK, which are separated by 40 residues inside the parental FGF-5 sequence. A second peptide, encoded by the melanoma differentiation Ag gp100<sup>PMEL17</sup> is made by the association of fragments RTK and QLYPEW, which are distant of 4 aa in the parent protein (25). The two other spliced peptides described were produced by re-
verse splicing, that is, splicing of peptide fragments in the reverse order to that in which they appear in the parental protein (26, 27). One of them is a minor histocompatibility Ag created by a poly-
morphism in the SP110 nuclear phosphoprotein gene and made after splicing of peptide segments STPK and SLPRTG to create the antigenic peptide SLPRTGSTPK (27). The fourth antigenic peptide, which is presented by HLA-A*0201 and recognized by a tumor-infiltrating lymphocyte isolated from a melanoma patient. Contrary to the previously described spliced peptides, this 9-aa peptide results from the association of an 8-aa peptide fragment with a single arginine residue. We provide a de-
tailed analysis of the different steps required for the production of this antigenic peptide.

Materials and Methods

Cell lines and tumor-infiltrating CTL

Part of a surgically excised cutaneous metastasis of patient M45 (HLA-A3, B7, B44, Cw5, Cw7) was used to establish the autologous melanoma cell line M45. This cell line was cultured in IMDM (Life Technologies, Carlsbad, CA) containing 10% FBS (Thermo Scientific). Another portion of the tumor was cultured in the presence of rIL-2 (R&D Systems, Minneapolis, MN), as described previously (29), to allow outgrowth of tumor-infiltrating T cells. This tumor-infiltrating T cell population was cloned under limiting dilution conditions, and stable antitumor CTL clones were selected and expanded by stimulation every 14 d with 100 U/ml IL-2. One of these clones was cloned under limiting dilution conditions, and stable antitumor CTL clones were selected and expanded by stimulation every 14 d with 100U/ml IL-2. This clone was cultured in IMDM (Life Technologies, Carlsbad, CA) containing 10% human serum (CTL medium), rIL-2 (150 U/ml), and IDO inhibitor 1-methyl-L-tryptophan (200 µM). Melanoma cell lines M17, M88, and M110 were obtained (30). Melanoma cell lines M17, M88, and M110 were cultured in IMDM containing 10% human serum (CTLMedium), and the maximum release from targets incubated with medium without antigen was measured by testing their cytotoxicity on WEHI 164 clone 13 in a colorimetric assay (9, 33, 34) or by ELISA for TNF-α (Fig. 4) (Life Technologies).

RT-PCR

RT-PCR was carried out as previously described (31). Briefly, total RNA from melanoma cell lines was extracted using the NucleoSpin RNAII kit (Machery-Nagel, Dueren, Germany) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase under conditions specified (25) in the kit manual (Life Technologies). cDNA samples were amplified (21 cycles of 1 min at 94°C, 1 min at 64°C, and 1 min at 72°C) with gp100 primers 5′-AGTCTAGGGGCCCCAGTGTCT-3′ and 5′-GGG-
GCCAGGCCCTGAGTAATGAT-3′. Aliquots of PCR products were size fractionated on agarose gels, and amplification products were visualized using ethidium bromide.

Transfection of COS-7 cells

Transfection into COS-7 cells was performed using the diethylaminoethyl-
dextran-chloroquine method, as described (32). In brief, 2.5 × 10^5 (1.5 × 10^6 in Fig. 4) COS-7 cells were cotransfected with 200 ng HLA-A*0301 cDNA (50 ng in Fig. 4) and 200 ng gp100-derived constructs (50 ng in Fig. 4). After 24 h, transfected cells were tested for their ability to stimulate TNF release by CTL M45-3B. Briefly, 1.5 × 10^6 CTL were added to the transfected cells in 200 µl CTL medium containing rIL-2 (25 U/ml). Coculture supernatant was collected after 16 h, and their TNF content was measured by testing their cytotoxicity on WEHI 164 clone 13 in a colorimetric assay (9, 33, 34) or by ELISA for TNF-α (Fig. 4) (Life Technologies).

The gp100-derived constructs used for transfection were designed as follows. The cDNA encoding HLA-A*0301 was cloned between the BstXI and NotI sites of pcDNA3. In Fig. 1B, a cDNA spanning the first 2009 bp of the published (35) gp100 cDNA sequence (extending 1985 bp beyond the initiation codon up to the Scal site) was subcloned between the EcoRI and NotI sites of pcDNA3 (Life Technologies). Various deletion mutants were obtained after digestion and religation of the gp100 sequence into pcDNA3 using Apal (gp1001–211) or Kpel (gp1005–85). Other truncated variants were generated by PCR amplification using the following couples of primers: 5′-gaatgggaggaagACCAATAGG-3′ and 5′-cttagCTAC-
TCGATTAGTGGTAAAG-3′, 5′-gaagaGCCACCTGAGATCCT-3′, and 5′-cttagCTACACAGCTGACCC-3′, and 5′-gaagaGCCAC-
CATGAGTGGTAAAG-3′ and 5′-cttagCTACACAGCTGACCC-3′ to produce the subgenic gp100 fragments encoding gp100184–211, gp10052–211, gp1005–211, respectively. Each forward primer contains an EcoRI restriction site (underlined), the consensus kozak sequence (italic), and ATG internal sequence codon (bold). The reverse primers all contain a XbaI restriction site (underlined) and a stop codon (bold). These truncated cdna fragments were all cloned into pcDNA3.

Site-directed mutagenesis

In Figs. 2A and 4, site-directed mutagenesis was carried out using the gp100184–211 minigene using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and the primers listed in Table I.

Peptide electroporation

A total of 2 × 10^8 HLA-A*0301 CAN-EBV B cells was electroporated, as previously described (25), in 0.4-cm cuvettes using 400 µl electroporation buffer (K2HPO4/KH2PO4 [10 mM; pH 7.5], MgCl2 [1 mM], su-
crose [250 mM]) and 100 µM peptide. Electroporation was performed using a GenePulser Xcell electroporator (Bio-Rad, Hercules, CA) functioning in square wave mode with 10 pulses of 0.1 ms at 480 V separated by 1-s intervals. Cells were diluted to a peptide concentration of 6.7 µM and incubated for 4 h in culture medium. Cells were washed and tested for their ability to activate IFN-γ production by CTL M45-3B, as follows: 5 × 10^5 electroporated CAN-EBV B cells were incubated overnight in a 96-well plate with 2.5 × 10^5 CTL. As a control, electroporated cells were loaded with antigenic peptide (2 µg/ml) for 1 h and washed. The pu-
duction of IFN-γ was measured by ELISA after an overnight incubation or by FACs staining, as previously described (36).

Peptide synthesis and recognition assay

Peptides were synthesized on solid phase using conventional fluorenyl-
methoxycarbonyl chemistry, purified by reverse-phase HPLC (>95% pu-
rity, and characterized by mass spectrometry. The lyophilized peptides were dissolved at 20 mg/ml in DMSO and stored at −20°C. Peptides were loaded onto 5 × 10^6 HLA-A*0301 CAN-EBV B cells at the indicated concentration (1 h at 37°C) before addition of the CTL M45-3B (15 × 10^6 cells/well). IFN-γ release was determined by ELISA (Life Technologies) after 16 h of incubation.

Cytotoxicity assays

Standard chromium release assays were performed, as previously de-
scribed (29), by incubating Na251CrO4-labeled (Perkin Elmer, Waltham, MA) target cells with the CTL at an E/T ratio of 10:1. After 4 h of incubation at 37°C, 25 µL supernatant was collected and added to 100 µL opthase supermix mixture (Perkin Elmer) before gamma counting. The percentage specific lysis was calculated as follows: (sample release − spontaneous release)/(maximum release − spontaneous release) × 100. The spontaneous release was obtained from targets incubated with medium, and the maximum release from targets incubated with medium containing 1% Triton X-100.

The Journal of Immunology 1963
Isolation and fractionation of MHC-bound peptides present on melanoma cells

Peptides were acid eluted from immunopurified HLA class I molecules from 4.4 × 10⁶ M45 melanoma cells, as described previously (25). Peptides were further fractionated by HPLC after injection on a 4.1 × 150-mm ACE Phenyl (Advanced Chromatography Technologies, Aberdeen, U.K.) at a flow rate of 0.9 ml/min with an elution gradient of 4–7% acetonitrile/0.1% formic acid (v/v) over 3 min, followed by 7–18% acetonitrile/0.1% formic acid over 30 min. Lyophilized fractions were resuspended in 20 μl water, diluted in 80 μl X-vivo 10 medium (Life Technologies), and loaded in duplicate onto 5 × 10⁶ HLA-A*0301 CAN-EBV B cells before addition of CTL M45-3B. The IFN-γ released in the supernatants was measured by ELISA (Life Technologies) after an overnight coculture.

HPLC and mass spectrometry

Samples were separated by reverse-phase chromatography on a PepMap C18 0.3/15 column (LC Packings, Sunnyvale, CA) and eluted using a 35-min linear gradient of acetonitrile in water (5–50%) containing 0.05% trifluoroacetic acid with a flow rate of 4 ml/min. Mass spectrometry analysis (MS) was performed online with a LCQ Deca XP Plus ion-trap mass spectrometer equipped with an electrospray ionization source (Thermo-Finnigan, San Jose, CA) and operated in positive mode with default parameters and active automatic gain control. Mass spectra were acquired in a mode that alternated single MS scans (m/z 300–2000) with tandem MS (MS/MS) scans.

Proteasome inhibition in melanoma cells

Cells were first acid eluted in a glycine buffer at pH 3 for 30 s to release MHC-bound peptides and then neutralized in culture medium. Cells were then treated for 1 h at 37˚C with 0, 125, or 250 nM epoxomicin (12.5 nM) in a 6-well plate. Cells were then washed again and treated for 1 h at 37˚C with 60, 125, or 250 nM epoxomicin, as indicated (Sigma-Aldrich), in X-vivo 10 medium (Bio-Whittaker, Walkersville, MD). Cells were then washed and incubated for 4 h with lower concentrations of epoxomicin (12.5 nM) in a 6-well plate. Cells were then washed again and tested for their ability to activate IFN-γ release by CTL M45-3B, as follows: 5 × 10⁴ epoxomicin-treated cells were incubated overnight in a 96-well plate with 25 × 10⁵ CTL. As a control, electroporated cells were loaded with antigenic peptide (2 μg/ml) for 1 h and washed.

Results

CTL M45-3B recognizes an unconventional HLA-A*0301–restricted peptide derived from the melanoma Ag gp100

By culturing fragments of a cutaneous melanoma metastasis with IL-2 in limiting dilution conditions, we isolated a CTL clone (M45-3B) that was able to lyse the autologous tumor line M45 as well as three allogeneic melanoma cell lines expressing HLA-A*0301 and gp100 (38) (Fig. 1A). Because the CTL clone did not lyse any of the HLA-A*0301+/gp100+ or HLA-A0301+/gp100− cell lines tested (data not shown and Fig. 1A), the peptide recognized by CTL M45-3B was most likely encoded by gp100 and presented by HLA-A*0301.

To identify the region of gp100 containing the peptide recognized by CTL M45-3B, we produced truncated gp100 cDNA fragments and cloned these cDNAs into expression vector pcDNA3. These constructs were transfected into COS-7 cells together with

![Figure 1](https://www.jimmunol.org/)
the HLA-A*0301 cDNA, and their ability to activate CTL M45-3B was tested in a TNF release assay (Fig. 1B). This showed that the peptide recognized by CTL M45-3B was localized within a 28-aa fragment spanning residues M184 to D211.

Potential peptide candidates contained in this 28-aa fragment were synthesized and tested for their ability to activate CTL M45-3B. Surprisingly, none of these peptides was recognized by the CTL, suggesting that the gp100-derived epitope recognized by CTL M45-3B might arise from a posttranslational modification (Supplemental Fig. 1A).

Identification of the peptide recognized by CTL M45-3B

To identify which residues in this 28-aa fragment (M184–D211) were important for immunogenicity, we produced a series of minigenes encoding the 28-aa precursor with alanine substitutions at every position (Table I). We and others reported the existence of antigenic peptides containing noncontiguous fragments of the parental protein, which are spliced together in the proteasome by transpeptidation (24–28). We therefore considered the possibility that the peptide recognized by CTL M45-3B could also be produced by peptide splicing. This was a tempting possibility as we noted that fragment 195–203, containing noncontiguous fragments of the parental protein, which was a tempting possibility as we noted that fragment 195–203, was potentially spliced peptides were synthesized one of the arginines present at position 191 or 192. To test this possibility, 96 potentially spliced peptides were synthesized and grouped into 15 pools (Supplemental Fig. 2A), five of which corresponded to a reverse splicing in the region 186–202. These 15 pools were loaded onto HLA-A*0301–positive CAN-EBV B cells and tested for CTL recognition (Supplemental Fig. 2B). Among the 15 pools tested, 7 were positive (red in Supplemental Fig. 2A). Each of these 7 positive pools was fractionated by HPLC, and the fractions were tested for CTL recognition as previously. Peptides present in each positive fraction were identified by mass spectrometry (red in Supplemental Fig. 2A), synthesized, purified, and tested for CTL recognition (Fig. 2B). One of them, peptide RSYVPLAH_R, was efficiently recognized by the CTL (50% activation at a concentration close to 50 nM). Longer peptides GSRSYVPLAH_R, SRSYVPLAH_R, and RSYVPLAH_R induced a 50% activation of the CTL at a peptide concentration close to 1 µM (Fig. 2B). Finally, peptide RSYVPL_YHR was also recognized by the CTL but required an even higher concentration of peptide (50% activation at a concentration between 1 and 3 µM). To determine which epitope candidate was naturally found at the surface of the autologous tumor, we acid eluted the MHC class I–linked peptides from M45 melanoma cells, separated the peptides by HPLC, and tested each fraction for recognition by CTL M45-3B (Fig. 2C). The positive fraction had a retention time of 11 min, which corresponded to the retention time of the peptide RSYVPLAH_R fractionated in the exact same conditions, suggesting that the 9-aa peptide RSYVPLAH_R is the natural antigenic peptide presented by M45 melanoma cells to the HLA-A*0301–restricted CTL M45-3B.

Peptide RSYVPLAH_R is spliced and reordered in the proteasome

We previously demonstrated that spliced antigenic peptides are produced by transpeptidation inside the catalytic chamber of the proteasome (25–28). This process involves the production of an acyl-enzyme intermediate between a peptide fragment and the hydroxyl group of the N-terminal threonine of the catalytic subunit. In the course of proteolysis, this acyl-enzyme intermediate is rapidly hydrolyzed by water molecules present in the catalytic chamber. During the peptide-splicing process, however, the N terminus of the acyl-enzyme intermediate becomes covalently linked to the MHC class I–linked peptide. We and others have previously shown that fragments corresponding to reverse splicing in the 186–202 region of gp100, whereas the 10 other pools corresponded to a reverse splicing in the region 186–202. These 15 pools were loaded onto HLA-A*0301–positive CAN-EBV B cells and tested for CTL recognition (Supplemental Fig. 2B). Among the 15 pools tested, 7 were positive (red in Supplemental Fig. 2A). Each of these 7 positive pools was fractionated by HPLC, and the fractions were tested for CTL recognition as previously. Peptides present in each positive fraction were identified by mass spectrometry (red in Supplemental Fig. 2A), synthesized, purified, and tested for CTL recognition (Fig. 2B). One of them, peptide RSYVPLAH_R, was efficiently recognized by the CTL (50% activation at a concentration close to 50 nM). Longer peptides GSRSYVPLAH_R, SRSYVPLAH_R, and RSYVPLAH_R induced a 50% activation of the CTL at a peptide concentration close to 1 µM (Fig. 2B). Finally, peptide RSYVPL_YHR was also recognized by the CTL but required an even higher concentration of peptide (50% activation at a concentration between 1 and 3 µM). To determine which epitope candidate was naturally found at the surface of the autologous tumor, we acid eluted the MHC class I–linked peptides from M45 melanoma cells, separated the peptides by HPLC, and tested each fraction for recognition by CTL M45-3B (Fig. 2C). The positive fraction had a retention time of 11 min, which corresponded to the retention time of the peptide RSYVPLAH_R fractionated in the exact same conditions, suggesting that the 9-aa peptide RSYVPLAH_R is the natural antigenic peptide presented by M45 melanoma cells to the HLA-A*0301–restricted CTL M45-3B.

Table I. Primers used for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Mutagenized site</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>E185A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GACATGCTACCGCATGGGCCG-3'</td>
</tr>
<tr>
<td>V186A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GACATGCTACCGCATGGGCCG-3'</td>
</tr>
<tr>
<td>T187A</td>
<td>5'-CCATGGAAGTGCCTACAC-3'</td>
<td>5'-CCATGGAAGTGCCTACAC-3'</td>
</tr>
<tr>
<td>V188A</td>
<td>5'-CCATGGAAGTGCCTACAC-3'</td>
<td>5'-CCATGGAAGTGCCTACAC-3'</td>
</tr>
<tr>
<td>Y189A</td>
<td>5'-CCATGGAAGTGCCTACAC-3'</td>
<td>5'-CCATGGAAGTGCCTACAC-3'</td>
</tr>
<tr>
<td>H190A</td>
<td>5'-ACTGCTATATGAGCTTCCGCCGAGC-3'</td>
<td>5'-ACTGCTATATGAGCTTCCGCCGAGC-3'</td>
</tr>
<tr>
<td>R191A</td>
<td>5'-ACTGCTATATGAGCTTCCGCCGAGC-3'</td>
<td>5'-ACTGCTATATGAGCTTCCGCCGAGC-3'</td>
</tr>
<tr>
<td>R192A</td>
<td>5'-ACTGCTATATGAGCTTCCGCCGAGC-3'</td>
<td>5'-ACTGCTATATGAGCTTCCGCCGAGC-3'</td>
</tr>
<tr>
<td>G193A</td>
<td>5'-ACTGCTATATGAGCTTCCGCCGAGC-3'</td>
<td>5'-ACTGCTATATGAGCTTCCGCCGAGC-3'</td>
</tr>
<tr>
<td>S194A</td>
<td>5'-ACTGCTATATGAGCTTCCGCCGAGC-3'</td>
<td>5'-ACTGCTATATGAGCTTCCGCCGAGC-3'</td>
</tr>
<tr>
<td>R195A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>S196A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>Y197A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>V198A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>P199A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>L200A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>H202A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>S203A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>S204A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>S205A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>F207A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>T208A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>I209A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>T210A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>D211A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
<tr>
<td>R191A-R192A</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
<td>5'-GCCACCATGCAAGTGATGCTC-3'</td>
</tr>
</tbody>
</table>
other peptides present in the chamber can compete with water molecules to produce a nucleophilic attack on the acyl-enzyme intermediate, thereby creating a new spliced peptide by transpeptidation.

To assess the role of the proteasome in the processing of the RSYVPLAH_R peptide, we treated the autologous melanoma cell line M45 with increasing concentrations of the specific proteasome inhibitor epoxomicin. This treatment decreased CTL recognition in a dose-dependent manner, suggesting that proteasome inhibition prevents processing of the RSYVPLAH_R peptide (Fig. 3A). To confirm the role of the proteasome in the reverse splicing, we incubated purified 20S standard proteasomes with the 17- or 18-aa precursors gp100192–208 or gp100 191–208. Digests were then pulsed onto HLA-A*0301 + CAN-EBV B cells and tested for CTL recognition. As expected, only digests containing proteasomes and the precursor peptides were recognized by CTL M45-3B (Fig. 3B), confirming the ability of the proteasome to produce the antigenic peptide from these precursor peptides.

Mechanisms involved in the splicing and reordering of peptide RSYVPLAH_R
To define the mechanism of production of spliced peptide RSYVPLAH_R, we first located the sites of proteasome cleavage using mass spectrometry to identify the fragments present in a digest obtained with a 22-aa precursor peptide (Fig. 3C). Interestingly, one of the cleavages was observed after residue H202, which is the last residue of the N-terminal fragment of the spliced peptide. This indicated that the proteasome was able to form the acyl-enzyme intermediate required for production of peptide RSYVPLAH_R. Multiple other cleavages were also observed around the arginines R191 and R192, which can compose the C-terminal residue of the spliced peptide.

We then set out to define which arginine residue was involved in the splicing reaction and contributed the C-terminal position of the antigenic peptide. The 28-aa–long precursor fragment contains three arginines, as follows: R191, R192, and R195. Because R195 corresponds to the N-terminal residue of the antigenic peptide and is therefore not expected to be involved in the production of the C terminus of the same peptide, we studied the involvement of the other two arginines, R191 and R192, which can compose the C-terminal residue of the spliced peptide.

We then set out to define which arginine residue was involved in the splicing reaction and contributed the C-terminal position of the antigenic peptide. The 28-aa–long precursor fragment contains three arginines, as follows: R191, R192, and R195. Because R195 corresponds to the N-terminal residue of the antigenic peptide and is therefore not expected to be involved in the production of the C terminus of the same peptide, we studied the involvement of the other two arginines, R191 and R192, which can compose the C-terminal residue of the spliced peptide.

FIGURE 2. Identification of the antigenic peptide recognized by CTL M45-3B. (A) Identification of the amino acids that are essential for antigenicity. COS-7 cells were transfected with the HLA-A*0301 cDNA and with vectors encoding the indicated gp100 peptide sequences. Alanine substitutions are shown with a bold A with a subscript indicating the amino acid position in the gp100 protein. TNF production by the CTL was measured after 18 h of coculture. Relative TNF production to nonmutated gp100 minigene (taken as 100%) is represented, with error bars showing SDs between two independent experiments. (B) Recognition of peptide RSYVPLAH_R by CTL M45-3B. HLA-A*0301 + CAN-EBV B cells were incubated with the indicated peptides. CTL M45-3B was added 60 min after peptide loading, and IFN-γ production by the CTL was measured after 24-h coculture. Error bars show SDs of duplicates. Data are representative of two independent experiments. (C) Elution of the natural peptide recognized by CTL M45-3B. Peptides eluted from purified HLA class I molecules of melanoma cells M45 were separated by HPLC, and the fractions were tested for recognition by CTL M45-3B (top panel). To rule out contamination of the HPLC system, buffer was run on the column before the eluted samples, and the fractions were tested similarly. Synthetic peptides RSYVPLAHRR and RSYVPLAH (250 ng each) were mixed and injected under the same HPLC conditions, and the fractions were tested for CTL recognition (middle panel). Peptides present in each fraction were analyzed by MS/MS and are indicated by the letter A (RSYVPLAHRR) or B (RSYVPLAH). The bottom panel shows the UV absorption spectrum of the peptides RSYVPLAHRR (A), RSYVPLAH (B), or RSYVPLHYR (C), which were injected under the same HPLC conditions. mAU, Milliabsorbance units.
either arginine could generate the C terminus of the antigenic peptide. Surprisingly, the substitution of both R191 and R192 did not completely abrogate peptide presentation (Fig. 4), suggesting that the C-terminal arginine could also originate either by splicing of R195 from a second molecule of the same peptide or by splicing of the N-terminal arginine from an unrelated peptide fragment. This observation was reinforced by the positive CTL response observed after electroporation of the RSYVPLAHR peptide into EBV-B cells (Supplemental Fig. 1B). In vitro, the generation of the antigenic peptide from the RSYVPLAHSSSAFT195–208 precursor by purified 20S proteasomes was also observed (Supplemental Fig. 3). It confirms our previous observation that the production of antigenic peptides can also occur by trans-splicing, that is, through the splicing of fragments originating from distinct molecules (28). Nevertheless, we observed that this phenomenon only minimally contributed to the production of peptides in physiological conditions (28). The experiment shown in Fig. 4 was performed using peptide-encoding minigenes overexpressed after transfection into COS-7 cells using a plasmid amplification system. These artificial conditions may explain why we detected trans-splicing in this experiment. To avoid the formation of the antigenic peptide by trans-splicing in our in vitro digestion experiments, we chose to acetylate the N-terminal arginine of each precursor peptide used in subsequent in vitro digestions, to prevent the nucleophilic attack of the acyl-enzyme intermediate (25).

This observation was reinforced by the positive CTL response observed after electroporation of the RSYVPLAHR peptide into EBV-B cells (Supplemental Fig. 1B). In vitro, the generation of the antigenic peptide from the RSYVPLAHSSSAFT195–208 precursor by purified 20S proteasomes was also observed (Supplemental Fig. 3). It confirms our previous observation that the production of antigenic peptides can also occur by trans-splicing, that is, through the splicing of fragments originating from distinct molecules (28). Nevertheless, we observed that this phenomenon only minimally contributed to the production of peptides in physiological conditions (28). The experiment shown in Fig. 4 was performed using peptide-encoding minigenes overexpressed after transfection into COS-7 cells using a plasmid amplification system. These artificial conditions may explain why we detected trans-splicing in this experiment. To avoid the formation of the antigenic peptide by trans-splicing in our in vitro digestion experiments, we chose to acetylate the N-terminal arginine of each precursor peptide used in subsequent in vitro digestions, to prevent the nucleophilic attack of the acyl-enzyme intermediate (25).

Production of the peptide RSYVPLAH_R involves the addition of single arginine to the peptide RSYVPLAHR. In an attempt to reproduce this reaction in vitro, we incubated 20S standard proteasomes with the precursor peptide Ac-RSYVPLAHR before the CTL assay to check their ability to present Ags. Relative activities (left panel) were calculated as a proportion of the activity measured with the synthetic peptide control. This proportion was then related to that observed without treatment, which was taken as 100%. The actual values of IFN-γ measured by ELISA are shown on the right panel. Error bars show SDs of triplicates. Data are representative of four independent experiments. (B) In vitro production of the gp100 spliced peptide by proteasomes. Purified 20S standard proteasomes were used to digest the 17- or 18-aa precursor peptides RGSRSYVPLAHSSSAFT195–208 and RRGSRGSRVPLAHSSSAFT191–208. Digests were then loaded on HLA-A*0301–positive CAN-EBV B cells and tested for recognition by CTL M45-3B. After an overnight coculture, IFN-γ production was measured by ELISA. Error bars show SDs of duplicates. Data are representative of three independent experiments. (C) Peptide fragments detected by mass spectrometry after digestion of 22-aa precursor VTYYHRGGRSRSYVPLAHSSSAFT186–207 by 20S standard proteasomes. N-terminal (blue) and C-terminal (red) fragments predicted to result from cleavages at every indicated position were searched by mass spectrometry in a 60-min digest. The experimental conditions did not allow the detection of fragment SAF. The detected cleavages are indicated by arrows in the lower panel. Data are representative of two independent experiments.

FIGURE 3. Role of the proteasome in the peptide splicing. (A) Proteasome inhibition prevents the production of the spliced gp100 antigenic peptide. Acid-eluted tumor cells M45 were treated with epoxomicin and tested for recognition by CTL M45-3B in an IFN-γ production assay. Where indicated, cells were loaded with synthetic peptide RSYVPLAHR before the CTL assay to check their ability to present Ags. Relative activities (left panel) were calculated as a proportion of the activity measured with the synthetic peptide control. This proportion was then related to that observed without treatment, which was taken as 100%. The actual values of IFN-γ measured by ELISA are shown on the right panel. Error bars show SDs of triplicates. Data are representative of four independent experiments. (B) In vitro production of the gp100 spliced peptide by proteasomes. Purified 20S standard proteasomes were used to digest the 17- or 18-aa precursor peptides RGSRSYVPLAHSSSAFT195–208 and RRGSRGSRVPLAHSSSAFT191–208. Digests were then loaded on HLA-A*0301–positive CAN-EBV B cells and tested for recognition by CTL M45-3B. After an overnight coculture, IFN-γ production was measured by ELISA. Error bars show SDs of triplicates. Data are representative of three independent experiments. (C) Peptide fragments detected by mass spectrometry after digestion of 22-aa precursor VTYYHRGGRSRSYVPLAHSSSAFT186–207 by 20S standard proteasomes. N-terminal (blue) and C-terminal (red) fragments predicted to result from cleavages at every indicated position were searched by mass spectrometry in a 60-min digest. The experimental conditions did not allow the detection of fragment SAF. The detected cleavages are indicated by arrows in the lower panel. Data are representative of two independent experiments.

FIGURE 4. Identification of the arginine involved in the peptide splicing. COS-7 cells were transiently transfected with HLA-A*0301 cDNA and vectors encoding the indicated gp100 fragments, where arginine 191 and/or 192 were replaced by alanine, as indicated. Transfected COS-7 cells were then tested for their ability to activate CTL M45-3B. TNF production was measured by ELISA after an overnight coculture. Error bars show SDs of quadruplicates. Data are representative of four independent experiments.
arginine for the splicing reaction (Fig. 5). However, we did not detect peptide Ac-RSYVPLAH_R in the digest by tandem mass spectrometry, suggesting that the splicing reaction required a fragment longer than one residue. We then incubated 20S standard proteasomes with the precursor peptide Ac-RSYVPLAHSSSAFT and arginine-containing fragments comprising 2, 3, or 4 aa (RRG191–192, RG192–193, RRG191–193, RGS192–194, or RRG191–194). Only digests performed with fragments larger than 2 aa contained the corresponding spliced products Ac-RSYVPLAH_RRG, Ac-RSYVPLAH_RGS, and Ac-RSYVPLAH_RRGS, suggesting that the peptide fragment producing the nucleophilic attack on the RSYVPLAH-containing acyl-enzyme intermediate must be at least three-residue long (Fig. 5). We then wondered whether the minimal length requirement for the C-terminal nucleophile was particular to our epitope or a more general constraint. We therefore digested pairs of peptides corresponding to precursor fragments of three other spliced peptides derived from gp100 or FGF-5 (Fig. 5) (24, 25). Interestingly, in all cases, peptide splicing could not be detected when it involved nucleophile fragments shorter than 3 or 4 aa. Longer fragments of up to 10 residues could also be spliced, with the exception of those longer than 8 aa in the case of the FGF-5 splicing. The latter observation might be sequence related, resulting from the peptide’s inability to accommodate the nucleophile-binding channel.

Thus, the splicing reaction produces C-terminally elongated peptides that may need a further trimming to produce the final antigenic peptide. To determine whether the proteasome was able to perform the final trimming step required for the release of the peptide RSYVPLAH_R, we incubated 20S standard proteasomes with peptides Ac-RSYVPLAH_RGS, Ac-RSYVPLAH_RRG, and Ac-RSYVPLAH_RRGS and searched the digests for the presence of the antigenic peptide Ac-RSYVPLAH_R by mass spectrometry (Fig. 6). The antigenic peptide was most efficiently produced from precursor Ac-RSYVPLAH_RGS, indicating that the proteasome can trim this spliced peptide to produce the final antigenic peptide. Peptide trimming was not observed in the absence of proteasome or when lactacystin was added in the digestion mixture.

From these observations, we conclude that the production of the antigenic peptide RSYVPLAH_R occurs in two steps (Fig. 7). In a first step, an elongated spliced peptide is produced by reverse splicing of an N-terminal arginine-containing peptide, such as RGS, onto an acyl-enzyme intermediate involving one catalytic subunit of the proteasome and fragment RSYVPLAH. The resulting elongated spliced peptide RSYVPLAH_RGS is then trimmed by the proteasome to produce the final antigenic peptide RSYVPLAH_R.

Discussion
In this study, we identified a new tumor antigenic peptide, RSYVPLAH_R, presented by HLA-A*0301 melanoma tumor cells.
and produced by the splicing of two noncontiguous fragments of the melanoma differentiation Ag gp100. Contrary to the other spliced peptides described to date, which were composed of spliced precursors, we observed that the antigenic peptide was produced at a low level by trans-splicing, using the N-terminal fragments each containing 3–6 aa (24–27), the peptide RSYVPLAHR is produced by the addition of a single arginine residue, we wondered whether this arginine residue originated from the splicing of a single amino acid or of a longer peptide fragment bearing an arginine at its N-terminal end. We therefore performed a series of proteasome digests assessing the splicing of C-terminal splice fragment of decreasing sizes. We could not detect splicing of a single amino acid, nor splicing of fragments shorter than three residues. This was also true for three distinct spliced peptides that we studied in a similar way. We conclude that the splicing requires a minimal size of three residues for the C-terminal spliced fragment acting as the nucleophile.

The efficiency of peptide splicing most probably depends on the ability of the nucleophile peptide fragment to compete with water molecules and attack the acyl-enzyme intermediate. Among the factors involved are the relative concentration and life span of the nucleophilic peptide in the catalytic chamber, the nucleophilicity of its N-terminal end, and its orientation relative to the ester bond of the acyl-enzyme intermediate. Constraints that might influence these parameters are the distance of the nucleophile from the ester bond of the acyl-enzyme intermediate and its positioning into the catalytic pocket. Among the factors that were suggested to influence the efficiency peptide splicing (39, 40). Ideal positioning might be better achieved with longer peptide fragments that would fit more tightly into the nucleophile-binding channel. This binding channel was recently suggested to be distinct from the primed substrate-binding channel, forming an additional pocket where the C-terminal reactant peptide would be accommodated awaiting for the splicing to take place (39). In line with our data, the sequence and length of the C-terminal nucleophile might therefore be critical for the binding into this pocket and the ability of peptides to perform splicing. Moreover, proper positioning of the nucleophile peptide in the binding channel might allow a better activation of the peptide N-terminal end by basic amino groups found in the pockets, increasing its nucleophilicity by attracting a proton, as it is the case for water molecules during proteolysis (40, 41).

The fact that the nucleophile needs to be at least 3 aa long implies that the production of the final antigenic peptide reported in this study requires a second round of proteinolysis, occurring after splicing. We did not formally exclude that this additional trimming step could be performed by another protease, but our data show that the proteasome itself is able to perform this trimming step. This final trimming could therefore take place in the wake of the splicing event, in the course of substrate progression inside the catalytic chamber. Alternatively, trimming might take place during a second round of proteasome degradation, once the extended spliced peptide has left the catalytic chamber. The probability that both splicing and trimming happen in the same sequence of events inside the proteasome seems higher, as the chance that the peptide reaches another catalytic subunit is higher when the fragment is already inside the chamber. Moreover, exit of the spliced precursor from the catalytic chamber would also increase the probability that it gets degraded before reaching the catalytic chamber again.

Studying the proteasome cleavage preferences of the extended spliced precursors, we observed that the antigenic peptide was produced preferentially from precursor RSYVPLAH_RGS rather...
than from precursors RSYVPLAH_RRG and RSYVPLAH_RRG. This suggests that cleavage of our precursor by the standard proteasome is more efficient when the P1' position corresponds to a glycine rather than an arginine residue. Contrasting results regarding the preference of the proteasome for glycine or arginine in position P1' were previously obtained (42, 43). This might be related to the fact that first, precursor peptides or proteins are different in nature and size, and second, cleavage preferences are also influenced by residues present in positions other than P1'.

To date, all the spliced antigenic peptides described contained a C-terminal partner peptide of 4–6 aa. In this work, we describe an antigenic peptide that only requires the addition of a single amino acid. Theoretically, this should increase the probability of production of this peptide, because whatever nucleophile peptide containing an arginine at its N-terminus and able to bind the proposed nucleophile-binding channel could be used to generate a spliced extended precursor. Our experiments show that production of the RSYVPLAH peptide could already rely on two different arginine residues. The only additional requirement is that this extended precursor still needs to be adequately trimmed to produce the final antigenic peptide.

The existence of spliced peptides generated by the final addition of a single amino acid further strengthens the notion that peptide splicing by the proteasome increases the diversity of the peptide repertoire presented by MHC class I molecules. Although only five antigenic peptides were identified to date, the number of spliced peptides effectively produced and presented at the cell surface might be much higher than originally expected, despite the low efficiency of the peptide-splicing reaction in vivo (25). This is supported by the works from Liepe et al. and Mishto et al. (39, 44), who revealed that the proteasome produces a large number of spliced peptides of adequate length for presentation on MHC class I molecules. The authors have no financial conflicts of interest.

Acknowledgments

We thank Aline Depasse, Bénédicte Tollet, and Luc Pilotte for technical assistance. We are grateful to Julie Klein and Suzanne Depelchin for help in the preparation of this manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


