Rapid and Sensitive Identification of Major Histocompatibility Complex Class I-associated Tumor Peptides by Nano-LC MALDI MS/MS*

Sandra Hofmann‡§, Matthias Glückmann¶, Sandra Kausche‖, Andrea Schmidt‡, Carsten Corvey‡, Rudolf Lichtenfels‖, Christoph Huber‖, Christian Albrecht¶, Michael Karas‡**, and Wolfgang Herr¶

Identification of major histocompatibility complex (MHC)-associated peptides recognized by T-lymphocytes is a crucial prerequisite for the detection and manipulation of specific immune responses in cancer, viral infections, and autoimmune diseases. Unfortunately immunogenic peptides are less abundant species present in highly complex mixtures of MHC-extracted material. Most peptide identification strategies use microcapillary LC coupled to nano-ESI MS/MS in a challenging on-line approach. Alternatively MALDI PSD analysis has been applied for this purpose. We report here on the first off-line combination of nanoscale (nano) LC and MALDI TOF/TOF MS/MS for the identification of naturally processed MHC peptide ligands. These peptides were acid-eluted from human leukocyte antigen (HLA)-A2, HLA-A3, and HLA-B/C complexes separately isolated from a renal cell carcinoma cell lysate using HLA allele-specific antibodies. After reversed-phase HPLC, peptides were further fractionated via nano-LC. This additional separation step provided a substantial increase in the number of detectable candidate species within the complex peptide pools. MALDI MS/MS analysis on nano-LC-separated material was then sufficiently sensitive to rapidly identify more than 30 novel HLA-presented peptide ligands. Peptide sequences contained perfect anchor amino acid residues described previously for HLA-A2, HLA-A3, and HLA-B7. The most promising candidate for a T-cell epitope is an HLA-B7-binding nonamer peptide derived from the tumor-associated gene NY- BR-16. To demonstrate the sensitivity of our approach we characterized peptides binding to HLA-C molecules that are usually expressed on the cell surface at approximately only 10% the levels of HLA-A or HLA-B. In fact, multiple renal cell carcinoma peptides were identified that contained anchor amino acid residues of HLA-Cw5 and HLA-Cw7. We conclude that the nano-LC MALDI MS/MS approach is a sensitive tool for the rapid and automated identification of MHC-associated tumor peptides. Molecular & Cellular Proteomics 4:1888–1897, 2005.

Cytotoxic T-lymphocytes (CTLs)1 recognize oligopeptides that are presented on the cell surface by MHC class I molecules (1). Usually these peptides are generated in the cytosolic proteasome compartment where endogenous proteins resulting from viral infection, cellular transformation, or tissue transplantation are digested by peptidase activity (2). TAP (transporter associated with antigen processing) proteins transport these peptide fragments into the endoplasmic reticulum (3). In the endoplasmic reticulum, peptides are loaded onto MHC class I molecules depending on certain requirements such as a final length of 8–14 amino acids and the presence of distinct anchor amino acid residues. Subsequently mature MHC-peptide complexes move to the cell surface where they can be recognized by CTLs. The identification of CTL-defined peptide epitopes occurring specifically in tumors, viral infections, or autoimmune disorders is an important step toward a comprehensive understanding of the interactions between the immune system and these diseases. In addition, it may allow for an improvement in the specificity and efficiency of immunotherapeutic strategies, particularly vaccination and adoptive CTL transfer (4).

Current strategies toward the molecular identification of naturally processed CTL epitopes have been developed by several MS and immunology groups during the last 2 decades (5). Briefly most procedures rely on the acid extraction of peptides from immunofinity-purified HLA-class I molecules with subsequent reduction of sample complexity by several rounds of HPLC purification. Resulting peptide fractions are screened with bioassays utilizing disease-associated CTL...
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clones to confirm and track the presence of relevant epitopes. In bioactive fractions, peptide epitopes are identified using μLC separation coupled on line with nano-ESI MS/MS analysis. It has been estimated that the natural peptide pool extracted from single class I MHC molecules contains more than 10,000 different species that are present in the range of 1–10,000 copies per cell (6). Despite these unfavorable parameters, clear improvements in instrument sensitivity, ionization efficiency, and sample handling techniques have made MS increasingly successful in identifying single epitopes within complex peptide mixtures (6–12). As a clear advantage over genetic antigen identification strategies, MS analysis allows for the identification of T-cell epitopes that result from post-translational modifications (13).

Nevertheless identification and characterization of naturally processed T-cell epitopes by μLC ESI MS/MS remains a challenging and delicate procedure, particularly if sample impurities and analyte suppression effects are present. Furthermore the peptide species of interest are only available for MS analysis during their column elution phase, and the sample is entirely consumed in the continuous electrospray ionization process. In contrast, the off-line coupling of μLC devices with a discontinuous ionization method, such as MALDI, overcomes this limitation. It has been demonstrated previously that sequencing of HLA peptide ligands can be achieved via MALDI PSD analysis (14–17). MALDI PSD, however, raises new problems because conventional PSD is time-consuming, and the mass accuracy of the observed fragment ion signals is limited. Additionally it does not allow for precise precursor ion isolation or for precisely controlled energy input to assist fragmentation (15, 18). With a combination of nano-LC and MALDI MS/MS, we recently identified a naturally processed renal cell carcinoma (RCC) peptide recognized by an HLA-A3-restricted CTL clone (19). In the current report, we describe the further development of this approach, allowing for the first comprehensive analysis of natural peptides binding not only to HLA-A and -B but also to weakly expressed HLA-C alleles on a human tumor.

EXPERIMENTAL PROCEDURES

Flow Cytometric Analysis—Cells were incubated for 15 min at 4 °C with the following murine monoclonal antibodies (mAbs): MA2.1, an anti-HLA-A2 IgG1 (20); GAP-A3, an anti-HLA-A3 IgG2a (21); BB7.1, an anti-HLA-B7 IgG1 (22); and B1.23.2, an anti-pan HLA-B/C IgG2a (23). Isotype-matched control Abs were included as controls. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG F(ab′)2 Ab (Immunotech, Marseille, France) was added for 15 min at 4 °C. Flow cytometry analysis was performed on an EPICS ALTRA™ flow cytometer using the software EXPOTM (Beckman Coulter, Fullerton, CA).

Extraction of Naturally Processed HLA-Class I-associated RCC Peptides—Naturally processed peptides were acid-eluted from purified HLA-class I molecules following a protocol described by Hunt et al. (9). Briefly renal clear cell carcinoma line MZ1257-RCC (HLA-class I type A′0201/A′0301, B′0702/B′4402, Cw′0501/Cw′0702) was expanded in RPMI 1640 medium supplemented with 10% (v/v) FCS (Invitrogen) in cell factory culture systems (Nunc, Roskilde, Denmark). The cell line had been established previously from tumor tissue of RCC patient MZ1257 (Prof. A. Knuth, University of Zürich, Switzerland, and the Ludwig Institute for Cancer Research, Zürich, Switzerland). MZ1257-RCC cells were lysed in 200 ml of buffered detergent solution (150 mM NaCl, 20 mM Tris-Cl, pH 8.0, 1% (v/v) CHAPS solution containing protease inhibitors iodoacetamide (100 μM), aprotinin (5 μg/ml), leupeptin (10 μg/ml), pepstatin A (10 μg/ml), phenylmethylsulfonyl fluoride (1 mM), ethylene-diaminetetraacetic acid (2 mM), and sodium azide (0.04%) for 60 min on ice. CHAPS and protease inhibitors were purchased from Roche Diagnostics. After centrifugation at 100,000 × g for 60 min at 4 °C, supernatant was passed through chromatography columns (Econo, Bio-Rad) filled with Sepharose CL-4B beads (Amersham Biosciences) coupled with either glycine (first column) or individual mAbs recognizing distinct HLA-class I alleles. Antibodies used were anti-HLA-A2 IgG2b BB7.2 (24), anti-HLA-A3 GAP-A3 (21), anti-HLA-B/C B1.23.2 (23), and anti-pan HLA-class I W6/32 IgG2a (25). Sepharose matrices were then treated with 0.2 mM acetic acid (pH 2.7) (Sigma) to release peptides from HLA molecules. Peptides were filtered through Centricon 10-kDa YM-10 membranes (Millipore, Bedford, MA) at 3500 × g for 5 h at 4 °C. Filters were concentrated by vacuum centrifugation (RVC-2-18, Christ, Osterode, Germany) and stored at −80 °C.

Two-dimensional Gel Electrophoresis of Immunoaffinity-purified HLA-Class I Complexes—Aliquots (125–150 μg/gel) of immunoaffinity-purified HLA-class I samples were resuspended in lysis buffer (7 M urea (AppliChem Biochemica, Darmstadt, Germany), 2 M thiourea (Sigma), 0.2 M dimethylbenzlammonium propane sulfonate (ICN Biochemicals, Eschwege, Germany), 1% (w/v) DTT (AppliChem Biochemica), 4% (w/v) CHAPS (AppliChem Biochemica), 0.5% (v/v) Pharmalyte™ (Amersham Biosciences), and a trace of bromphenol blue dye (Serva, Heidelberg, Germany)). The lysates were subjected to sonification (2 × 5 cycles, 0.5 s at 100% power, UW 2070 sonicator, MS 73 needle; Bandelin, Berlin, Germany). Isoelectric focusing, second dimension SDS-PAGE separation, gel staining with colloidal Coomassie Blue, silver staining, and gel documentation were performed as described previously (26, 27). For gel analyses, the ProteinWisher software package (Version 2.1; Definiens AG, Munich, Germany) was used.

In-gel Digestion and Peptide Mass Fingerprint—For peptide mass fingerprinting and subsequent analysis, gels were sliced and subjected to an in-gel protocol (28). Mass spectra were recorded on a MALDI-TOF instrument (Voyager-DE™ STR, Applied Biosystems, Framingham, MA) at 3500 × g for 5 h at 4 °C.

Nano-LC Separation Coupled on Line with MALDI Target Spotting of MHC Peptide Ligands—HLA-class I-associated peptides were fractionated on a microbore C18 column (2.1 × 150 mm, 5-μm particles, 300-Å pore size; Vydac, Hesperia, CA) on a RP-HPLC instrument (HP1100; Agilent Technologies, Palo Alto, CA). The elution gradient used was 0–10% solvent B for 5 min, 10–75% B for the next 65 min, and 75–100% B for the next 5 min where solvent A was 0.1% (v/v) TFA (Sigma) in water, and solvent B was H2O/acetonitrile/TFA (40:60:0.0085, v/v/v). All water used was double distilled. ACN was purchased from Carl Roth (Karlsruhe, Germany). Fractions were collected every 60 s at a flow rate of 250 μl/min.

Nano-LC Separation Coupled on Line with MALDI Target Spotting of Candidate and Synthesized Peptides—HPLC-fractionated peptides derived from 6 × 104 RCC cell equivalents were lyophilized and dissolved in H2O/ACN/TFA (97:3:0.1, v/v/v). Subsequently these natural peptide mixtures as well as individual synthesized candidate peptides were fractionated by nano-LC either on an 1100 Nanoflow Proteomics Solution system (Agilent Technologies) or an Ultimate system ( Dionex, Sunnyvale, CA). With the Agilent Technologies instrument, samples were trapped on a Zorbax 300 SB precolumn (0.3 × 5 mm, 5 μm; Agilent Technologies) with a flow rate of 20 μl/min.
and a solvent composition of H₂O/ACN/TFA (97:3:0.1, v/v/v). Separation was performed on a Chromolith™ CapRod nanoscale C₁₈ column (100 μm × 150 mm; Merck) using H₂O/ACN/TFA (97:3:0.1, v/v/v) as solvent A and 0.1% (v/v) TFA in ACN as solvent B at a flow rate of 300 nl/min. The elution gradient was 3–10% B for 5 min, 10–20% B for 10 min, 20–30% B for 35 min, 30–70% B for 20 min, 70–90% B for 10 min, 90% B for 5 min, and 3% B for 10 min. Fractions were spotted onto 100-well stainless steel MALDI targets (Applied Biosystems) via the 1100 Series LC Micro Collection/Spotting System™ (Agilent Technologies). α-Cyano-4-hydroxycinnamic acid (Fluka, Buchs, Switzerland) was used as matrix at a concentration of 11 mM and dissolved in H₂O/ACN/TFA (30:70:0.1, v/v/v). The matrix was added to the eluent flow via a micro-T-piece (Upchurch, Oak Harbor, WA) at a flow rate of 1 μl/min. Spotting was started 10 min after initiation of the elution gradient.

On the Ultimate instrument, HPLC-purified peptides were fractionated with a PepMap™ nanoscale C₁₈ column (75 μm × 150 mm, 3 μm; Dionex) at a flow rate of 300 nl/min and a PepMap™ 100 precolumn (0.3 × 5 mm, 5 μm; Dionex) at a flow rate of 20 μl/min. The buffers and gradient system were identical to those described in the previous paragraph. The UV chromatogram was recorded at a wavelength (λ) of 214 nm. Peptide fractionation was performed with a Probot™ microfraction collector (Dionex). The α-cyano-4-hydroxycinnamic acid (Fluka) matrix was continuously added to the column eluent as described above. Again spotting was started 10 min after initiation of the elution gradient.

Screening and Identification of Candidate Peptides by MALDI Mass Spectrometry—The HPLC-fractionated peptide components were characterized by their molecular mass on a mass spectrometer using the MALDI-TOF instrument (Voyager-DE STR, Applied Biosystems). Individual samples contained natural peptides obtained from 6 × 10⁶ RCC cells equivalents and dissolved in H₂O/methanol/acetic acid (50:50:1, v/v/v) (Sigma).

Nano-LC fractions spotted on line onto the MALDI targets were analyzed in MS mode using the 4700 Proteomics Analyzer (Applied Biosystems) MALDI-TOF/TOF™ instrument equipped with a neodymium:yttrium-aluminum-garnet laser emitting at 355 nm with a repetition rate of 100 Hz. The MS spectra were processed using the Peak Explore™ (Applied Biosystems) software allowing non-redundant and fully automated selection of precursors for MS/MS acquisition. Peaks with a signal-to-noise ratio above 35 were selected automatically. After generating an extracted ion chromatogram for each possible precursor mass the MALDI target position was selected automatically for each signal showing the highest peak intensity. A mass filter excluding noise peaks and matrix cluster ions according to published rules (30, 31) was applied. CID MALDI MS/MS mass spectra were recorded on selected candidate peptides.

All MS and MS/MS spectra were recorded in positive ion reflector mode. For the Voyager-DE STR instrument, 1000 MS spectra were typically accumulated. For the 4700 Proteomics Analyzer, 1000 laser shots were typically accumulated in MS mode, whereas in MS/MS mode spectra up to 5000 laser shots were acquired and averaged. External calibration was performed using a mixture of peptides with known masses in MS mode (Sequazyme™ peptide mass standard kit, Applied Biosystems). MS spectra of the 4700 Proteomics Analyzer were recalibrated internally based on one matrix ion signal (m/z 877.03) giving mass accuracies in MS mode better than 35 ppm. MS/MS spectra in CID mode were externally calibrated using known fragment ion masses and ion signals observed in the MS/MS spectrum of angiotensin I (Sequazyme™ peptide mass standard kit, Applied Biosystems).

Resulting data were analyzed by GPS Explorer™ (Applied Biosystems) software, which invoked a MASCOT (Matrix Science, London, UK) database search using a human subset of the National Center for Biotechnology Information (NCBI) database, downloaded on July 16, 2004, to determine candidate peptides. Mass tolerance for the precursor ion was set to 50 ppm; mass tolerance for the fragment ions was 0.2 Da. MASCOT does not allow for the mass accuracy of fragment ions to be set in ppm units, thereby interfering with the statistical significance of the results. Therefore, manual validation was performed for each MS/MS spectra. Fragment ions of the identified peptides had a mass accuracy less than 100 ppm. Furthermore de novo sequencing was applied using DeNovo Explorer™ (Applied Biosystems) software with mass tolerance set to 0.2 Da, no fixed modifications, and no possible modifications selected.

As an additional quality control, all MASCOT-derived peptides had to contain the anchor amino acid residues known for the HLA-class I alleles expressed by the MZ1257-RCC tumor line. Usually these anchor amino acid residues are located at position 2 and/or position 9. For HLA-A*0201, for example, anchor amino acids are leucine or methionine at position 2 and valine or leucine at position 9. The SYFPEITHI database (32) at www.syfpeithi.de was used to obtain information on anchor amino acid residues of relevant HLA-class I alleles.

MASCOT peptide hits with a MOWSE score higher than 35 and an E-value less than 0.05 regularly contained amino acids at anchor positions fitting to the SYFPEITHI results. Peptide hits with a score of less than 20 were not considered. Peptide hits with a score between 20 and 35 that contained the correct anchor amino acid residues were additionally validated. These peptides were synthesized, and the MS/MS spectra of the synthesized peptides were compared with the MS/MS spectra that were acquired from the naturally processed peptides in the nano-LC MALDI MS/MS experiment. In addition, the elution behavior of synthesized peptides was verified using the identical LC conditions.

Peptide Synthesis—Peptides were synthesized using solid-phase Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry on a Syro peptide synthesizer (MultisynTech, Witten, Germany). They were purified to greater than 95% homogeneity by RP-HPLC and characterized by MS/MS after dilution to a concentration of 1 pmol/μl.

RESULTS

 Sequencing of Synthetic T-cell Epitopes Using Different Mass Spectrometry Instruments—We first evaluated different combinations of ionization methods and mass analyzers for their capability to generate sequence information on known oligopeptides recognized by CTLs in association with HLA-class I. Five synthetic nonamers corresponding to naturally occurring viral and tumor epitopes were chosen as model compounds and analyzed in positive ion mode using different MALDI and ESI mass spectrometer instruments (33). The resulting fragment ion spectra were used for a MASCOT database search. In our experience, MALDI-TOF/TOF mass spectrometry proved to be well suited for this application because of a high number of sequence-specific fragment ion signals and a precise determination of precursor ion and fragment ion masses (data not shown). We therefore chose this approach to characterize unknown naturally processed peptide ligands that were extracted from purified HLA-class I complexes of the human RCC cell line MZ1257-RCC. The entire workflow is demonstrated in Fig. 1.

 Reduced Expression of HLA-B/C Compared with HLA-A Alleles by the MZ1257-RCC Tumor—According to genomic HLA typing results, the RCC cell line MZ1257-RCC encodes HLA-A*0201, HLA-A*0301, HLA-B*0702, HLA-B*4402, HLA-
Cw*0501, and HLA-Cw*0702 (19). We first performed cell surface staining analysis using mAbs recognizing exclusively HLA-A2, HLA-A3, HLA-B7, or a common determinant of all HLA-B/-C molecules. Compared with autologous B-lymphocytes isolated from the MZ1257 patient, the MZ1257-RCC tumor demonstrated reduced expression levels for all of the HLA-class I alleles analyzed (Fig. 2). In addition, MZ1257-RCC expressed HLA-A2 and HLA-A3 at a much higher level than HLA-B and -C. After large scale expansion of the MZ1257-RCC line to \(\frac{2.5}{10^{10}}\) cells, a detergent lysate was prepared. From \(\frac{5}{10^{10}}\) cell equivalents, we first isolated total HLA-class I complexes using mAb W6/32, which recognizes a common determinant of all HLA-I molecules. Subsequently this immunoprecipitate was fractionated by two-dimensional gel electrophoresis. Resulting gel spots were analyzed using MALDI-TOF MS and demonstrated frequent signals for HLA-A2 and HLA-A3 molecules, whereas HLA-B7 complexes were rarely observed. In contrast, HLA-B44 and both HLA-C alleles were not detected (data not shown). This observation confirmed our cell surface staining results showing reduced protein expression levels for entire HLA-B/-C compared with HLA-A molecules in the MZ1257-RCC tumor line.

Separate Isolation of Tumor Peptides Binding to HLA-A2, HLA-A3, and HLA-B/C Molecules—From the remaining MZ1257-RCC lysate containing \(\frac{2.5}{10^{10}}\) cell equivalents, HLA-A2, HLA-A3, and HLA-B/C complexes were separately isolated using allele-specific mAbs. A total of 400 \(\mu\)g of HLA-A2, 250 \(\mu\)g of HLA-A3, and 300 \(\mu\)g of HLA-B/C molecules were obtained. HLA-A2-, HLA-A3-, and HLA-B/C-associated peptides were sequentially acid-eluted, filtered through a 10-
kDa cut-off device, and fractionated by RP-HPLC on a micro-bore C18 column. As a representative example, Fig. 3 shows the HPLC chromatogram on natural HLA-A3-derived tumor peptides.

Detection of HLA-Class I-associated RCC Peptides Using Nano-LC and MALDI-TOF MS—With the use of MALDI-TOF MS, we first screened individual RP-HPLC fractions for the presence of appropriate peptide species. In fraction number 32 of HLA-B/-C-associated RCC peptides, only 20 mass species with a signal-to-noise ratio of 10 or higher were identified within the 900–1350 m/z range that usually contains HLA-class I-binding peptide ligands. We therefore fractionated HPLC-separated peptide material on a nano-LC system using a nanoscale C18 column at a flow rate of 300 nl/min. Indeed this approach strongly increased the number of peptide species detectable in individual HPLC fractions. In fraction 32, the total number of peptides with m/z between 900 and 1350 increased to 55 after nano-LC separation, reflecting the separation and concentration effects of the nano-LC device. In line with published data (34), the additional LC separation step overcomes the effects of ion suppression in MALDI mass spectrometry of complex peptide mixtures. As the same preparation was used, the artificial degree of oxidation on methionine and tryptophan was expected to be identical in MALDI MS measurements prior to and after nano-LC separation. Nevertheless, peptide candidates with oxidized methionine or tryptophan were not included in our analysis.

Identification of Nano-LC-purified HLA-presented RCC Peptides by MALDI MS/MS—Nano-LC-separated peptides were characterized using an offline MALDI MS/MS approach. Resulting fragment ion spectra demonstrated a high number of sequence-specific signals. MS/MS spectra were submitted to MASCOT database search using a human subset of the NCBI database. In addition, MS/MS data were subjected to de novo sequencing using DeNovo Explorer software. As a representative example, the candidate ion of m/z 1293.54 observed in nano-LC-purified HPLC fraction number 32 retrieved the peptide sequence KYFDEHYEY belonging to human CDC28 protein kinase 2 protein, residues 11–19 (NCBI accession number gi/H208414502859) with the MOWSE score of 71 according to a MASCOT database search (Fig. 4A). Also de novo sequencing using the DeNovo Explorer software yielded peptide KYFDEHYEY. In addition, the naturally processed peptide obtained from the m/z 1293.54 ion and the synthetic peptide KYFDEHYEY demonstrated an identical fragmentation pattern clearly confirming the validity of our results (Fig. 4B). As a further control, the synthetic peptide KYFDEHYEY showed a retention time identical to that of the naturally occurred counterpart as determined using the nano-LC MALDI target spotting in combination with MALDI MS (data not shown). Peptide KYFDEHYEY contains tyrosine at positions 2 and 9, which are the main anchor amino acid residues of HLA-Cw*0702 (32) expressed by the MZ1257-RCC cell line. Taken together, we concluded that this peptide is a naturally processed HLA-Cw7 ligand of the MZ1257-RCC cell line.

The established nano-LC MALDI MS/MS strategy was subsequently applied to a total of six individual HPLC fractions containing peptide ligands from MZ1257-RCC-derived HLA-A2, HLA-A3, and HLA-B/-C molecules. From the HLA-B/-C-associated ligands, the candidate peptide ion of m/z 950.61 retrieved peptide APRVPVQAL with a MOWSE score of 31 according to a MASCOT database search (Fig. 5A). This peptide belongs to the serologically defined breast cancer antigen NY-BR-16, residues 744–752 (NCBI accession number gi/12060822). The naturally processed peptide obtained from the m/z 950.61 and the synthetic peptide APRVPVQAL dem-

**Fig. 2. Reduced HLA-class I expression on renal cell carcinoma line MZ1257-RCC.** Flow cytometric analysis was performed on MZ1257-RCC tumor cells (A) and MZ1257-EBV B-lymphocytes (B), both isolated from the MZ1257 patient (HLA-class I type: HLA-A2/A3, HLA-B7/B44, HLA-Cw5/Cw7). mAbs recognizing exclusively HLA-A2 (MA2.1), HLA-A3 (GAP-A3), HLA-B7 (BB7.1), or the entire HLA-B/-C alleles (B1.23.2) were used. Allele-specific mAbs for HLA-B44, HLA-Cw5, and HLA-Cw7 were not available. IgG isotype Abs served as controls. Data represent percentage of mean fluorescence expression level.

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Fig. 3. Reversed-phase HPLC separation of naturally processed HLA-A3-associated tumor peptides. Peptides were acid-eluted from HLA-A3 molecules purified from a detergent lysate of $2.5 \times 10^{10}$ cells of renal cell carcinoma line MZ1257-RCC. After 10-kDa cut-off filtration and subsequent concentration in a vacuum centrifuge, the peptide pool was fractionated by RP-HPLC on a microbore C$_{18}$ column using 0.1% (v/v) TFA in water as solvent A and H$_2$O/acetoniitrile/TFA (40:60:0.085, v/v/v) as solvent B. The UV spectrum was obtained at $\lambda = 214$ nm. Resulting data are shown in the 5–55-min range with retention of selected peaks marked. mAU represents milliabsorption units.

Fig. 4. Characterization of an HLA-Cw7-associated tumor peptide ligand by MALDI MS/MS. Peptide material from HPLC fraction 32 corresponding to $6 \times 10^{5}$ MZ1257-RCC cell equivalents was separated by nanoscale LC prior to automated fragmentation by the 4700 Proteomics Analyzer MALDI-TOF/TOF instrument. The total fragmentation procedure including database search lasted 3 h. Fragment ion signals are labeled as suggested by Roepstorff and Fohlman (48). A, CID mass spectrum was recorded on candidate peptide ions of m/z 1293.54 at spot position 67. The MASCOT database search retrieved peptide KYFDEHYEY with a MOWSE score of 71. The actual mass accuracy for the peptide was 17 ppm; it was 70 ppm for the fragment ion signals. There was no other significant peptide hit, and no further peptide was suggested with a score higher than 20. B, corresponding fragmentation pattern on 1 pmol/µl solution of synthetic peptide KYFDEHYEY.

Fig. 5. Peptide APRVPVQAL encoded by tumor antigen NY-BR-16 is a natural HLA-B7 ligand. This peptide was identified in HPLC fraction number 31 containing HLA-B/-C-eluted MZ1257-RCC peptide material. The nano-LC MALDI MS/MS approach was performed exactly as described in Fig. 4. A, CID mass spectrum recorded on candidate peptide ion of m/z 950.61 at spot position 43. The MASCOT database search retrieved peptide APRVPVQAL with a MOWSE score of 31. The actual mass accuracy for the peptide was 35 ppm; it was 75 ppm for the fragment ion signals. There was no other significant peptide hit, and no further peptide was suggested with a score higher than 20. B, corresponding fragmentation pattern on 1 pmol/µl of synthetic peptide APRVPVQAL.

onstrated an identical fragmentation pattern clearly confirming the validity of our results (Fig. 5B). Also de novo sequenc-
sequence APRVPVQAL. In accordance with our fragmenta-
sequence results, natural and synthetic peptides APRVPVQAL dem-
onstrated an identical elution behavior using the nano-LC MALDI MS approach (data not shown). Peptide APRVPVQAL con-
tains proline at position 2 and leucine at position 9, both of
which are the main anchor amino acid residues for HLA-
A2 and HLA-B8), we identified peptides not only by HLA-A2, HLA-A3, HLA-B7 but even by HLA-Cw5
and HLA-Cw7. To our knowledge, this is the first report dem-
onscstrating the successful identification of naturally processed
peptide ligands that associate with HLA-C on a tumor. Con-
dering the successful identification of naturally processed
peptide ligands present in six different RP-HPLC fractions
were identified regardless of whether they contained hydro-
phobic or hydrophilic amino acid residues (Table I). This is a
higher sequencing efficiency than previously described by
Flad et al. (15) using a MALDI PSD approach. These authors
obtained from a human RCC tumor line complete sequence
information on 14 peptides by analyzing 17 different RP-
HPLC fractions. In addition, whereas Flad et al. (15) identified
natural peptides binding to two different HLA alleles (i.e.
HLA-A2 and HLA-B8), we identified peptides presented not
only by HLA-A2, HLA-A3, and HLA-B7 but even by HLA-Cw5
and HLA-Cw7. To our knowledge, this is the first report demon-
strating the successful identification of naturally processed
peptide ligands that associate with HLA-C on a tumor. Con-
dering that HLA-C is expressed at the cell surface at a much
lower level (i.e. about 10%) than HLA-A or HLA-B (36), our
success in characterizing HLA-C ligands underlines the sen-
sitivity of our nano-LC MALDI MS/MS approach.

**DISCUSSION**

Here we report on the first successful combination of
nano-LC and MALDI-TOF/TOF MS for a sensitive and rapid
sequencing of naturally processed HLA-class I-associated
peptides. From a single RCC tumor line, more than 30 novel
peptide ligands present in six different RP-HPLC fractions
were identified regardless of whether they contained hydro-
phobic or hydrophilic amino acid residues (Table I). This is a
higher sequencing efficiency than previously described by
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sitivity of our nano-LC MALDI MS/MS approach.

**Table I**

Natural HLA-class I peptide ligands of human MZ1257-RCC tumor identified by nano-LC MALDI MS/MS

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a Experimental monoisotopic mass of (non-charged) peptide.

b Calculated monoisotopic mass of (non-charged) peptide.
In line with previous ESI work published by Weinschenk et al. (37), the majority of HLA-class I-extracted RCC peptides were derived from housekeeping genes with ubiquitous expression in normal and malignant tissues. These included the plethora of proteins involved in cell metabolism and growth with the main emphasis on ribosomal constituents. Apparently class I-presented RCC ligands contain numerous self-peptides that seem to reflect a comprehensive summary of the proteome of normal cells (38). Other peptide ligands were processed from proteins that are known to be overexpressed in cancer. Examples in this category were the CDC28 protein kinase 2 recently identified as an up-regulated gene in pancreatic cancer (39) and the elongation factor 1-γ described as an overexpressed gene in hepatocellular carcinoma (40). Of particular interest, we identified the first peptide encoded by the tumor-associated gene NY-BR-16. This antigen was originally identified by immunoscreening of breast cancer-derived cDNA expression libraries with serum immunoglobulin from breast cancer patients (41). Peptide ligands encoded by overexpressed genes may potentially serve as suitable target structures of HLA-class I-restricted T-cells in RCC and other tumors. Therefore, we are currently trying to raise specific T-cells against these candidate epitopes by in vitro stimulation of peripheral blood lymphocytes from healthy donors and RCC patients. If successful, we will investigate whether these peptide-specific T-cells recognize antigen-bearing tumors expressing the restricting HLA allele. This should ultimately help to prove their hypothetical utility for tumor immunotherapy.

μLC combined with tandem ESI MS in an on-line approach is the current gold standard for identification of MHC class I- and class II-presented T-cell epitopes (9, 42–44). Compared with LC ESI MS/MS, the off-line nano-LC MALDI MS/MS technique has the major advantage that the sample of interest can be repeatedly analyzed with minimal consumption. Therefore, retrospective confirmation of sequence composition by means of a more detailed fragmentation study is easy to accomplish. Because MALDI analysis usually consumes only a small proportion of the samples, the majority of peptide material is left for functional assays. There appears to be an advantage over the alternative MALDI PSD technique that frequently achieves only partial sequence coverage on peptides of interest (15, 17).

To the best of our knowledge, a comparative study on naturally processed HLA-presented tumor peptides using LC MALDI and LC ESI has not been published. Rather than considering MALDI and ESI techniques as competitive, they should be regarded as complementary because both approaches typically produce differing parent ion repertoires for complex peptide mixtures (5, 45). In a previous study concerning protein analysis using LC MALDI and LC ESI, 63% of all identified proteins were identified by both approaches (45). An additional 21% were identified using the LC MALDI MS/MS technique, and another 16% were identified using the LC ESI MS/MS approach. Therefore, combining data from MALDI and ESI analysis still remains a means of obtaining a more comprehensive and informative data set.

One limitation in the analysis of MHC-bound peptides is operator bias in selecting peptides from extremely complex mixtures for MS/MS. Current MALDI-TOF/TOF instruments, such as the 4700 Proteomics Analyzer used herein, are capable of acquiring large data sets in a very short time period. This allows for identification of sequences of many more peptides because of much higher throughput analysis in an operator-independent automated mode (5). In our study, a full characterization of each fraction was obtained within only a 3-h MS acquisition time including MS/MS and database searching. This demonstrates the power of the MALDI MS/MS instrumentation. Nevertheless the determination of precise sequence information on single peptide species within complex mixtures can be difficult due to suppression of peptide ionization as well as the occurrence of peptides with similar m/z (46). This limitation is usually overcome by separating the sample of interest by μLC prior to MS analysis. The successful coupling of nano-LC with a MALDI instrument for the identification of proteins has been described recently (47). We introduce herein the second dimension nano-LC separation of RP-HPLC-fractionated peptide material prior to characterization by a MALDI MS/MS instrument. This approach was sufficient to reduce the complexity of the crude peptide mixtures, thereby overcoming the effects of signal suppression and overlapping precursors usually observed in one-dimensional HPLC separations.

In conclusion, our study demonstrates that the off-line coupling of nano-LC with a MALDI-TOF/TOF mass spectrometer allows for the sensitive and automated characterization of naturally processed HLA-presented tumor peptides. Sequence analysis revealed that HLA-class I peptide ligands extracted from a human RCC tumor are either encoded by genes that are ubiquitously expressed in normal and malignant tissues, or they are derived from overexpressed cancer-related genes. Of the latter category, several novel peptides, such as the nonamer encoded by the tumor-associated gene NY-BR-16, are interesting candidates with potential applicability in tumor immunotherapy.

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§ To whom correspondence may be addressed. Tel.: 49-69-798-29916; Fax: 49-69-798-29918; E-mail: hofmann@iachem.de.

* To whom correspondence may be addressed. Tel.: 49-69-798-29916; Fax: 49-69-798-29918; E-mail: karas@iachem.de.
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