High Immunogenicity of the Human Leukocyte Antigen Peptidomes of Melanoma Tumor Cells

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Background: The antigenicity of cells is defined by HLA-bound peptides.
Results: Peptidomes of melanoma cells were characterized, and peptide sequences were determined by mass spectrometry.
Conclusion: Cellular peptidomes are highly heterogeneous and immunogenic.
Significance: The results define tumor antigenicity in melanoma and extend the understanding of antigen processing; the identified peptides are suited for cancer vaccines and developing improved algorithms for T cell epitope prediction.

Human leukocyte antigens (HLA) bind peptides generated by limited proteolysis in cells and present them at the cell surfaces for recognition by T cells. Through this antigen presentation function they control the specificity of T cell responses and thereby adaptive immune responses. Knowledge of HLA-bound peptides is thus key to understanding adaptive immunity and to the development of vaccines and other specific immune intervention strategies. To gain insight into the antigenicity of melanomas, peptides were extracted from HLA isolated from the tumor cells, separated by two-dimensional HPLC, and sequenced by mass spectrometry. The spectra were analyzed by database-dependent MASCOT searches and database-independent de novo sequencing and, where required, confirmed with synthetic peptides, which were also used to determine their immunogenicity. Comparing four different melanoma cell lines, little overlap of the HLA-bound peptides was found, suggesting a high degree of individualization of the HLA peptidomes. This notwithstanding, the peptidomes were highly immunogenic in the patients from whom the tumor cells had been established and in unrelated patients. This broad cross-patient immunogenicity was only exceptionally related to individual peptides. The majority of the identified epitopes were derived from low to medium abundance proteins, mostly involved in sensitive cellular processes such as cell cycle control, DNA replication, control of gene expression, tumor suppressor function, and protein metabolism. The peptides thus provide insights into processes potentially related to tumorigenesis. Furthermore, analyses of the peptide sequences yield information on the specificity of peptide selection by HLA applicable to the developing prediction algorithms for T cell epitopes.

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** The abbreviations used are: HLA, human leukocyte antigen(s); PBMC, peripheral blood mononuclear cells; VGF, vascular endothelial growth factor.
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HLA-bound peptides represent the protein turnover of a cell, which may differ from the steady-state expression levels of the proteins, the proteomes. Actually, comparisons of HLA peptidomes and proteomes of the same cells have shown limited correlation (1). The HLA-I peptidomes are thought to reflect the dynamics of the proteomes, i.e. the expression and turnover of the cellular proteins. Thereby, they are sensitive indicators of physiological and pathological states of the cell. Peptides bound by HLA are protected from further degradation so that also fragments of low expression proteins may accumulate to become efficient stimulators of T cellular immune responses.

Through combination of highly resolving HPLC and mass spectrometry (MS), it has become possible in recent years to analyze in great detail the peptidomes, i.e. the peptides bound by the HLA of particular cell types of particular individuals. Such analyses can provide information on pathological processes in cells and reveals how the immune system senses the state of the cells. HLA peptidome analyses thus may lead to new targets for immunotherapy or prophylactic vaccination. Here, we report the first analysis of the HLA-I peptidomes of melanoma tumor cells.

**EXPERIMENTAL PROCEDURES**

*Cell Lines*—Four melanoma cell lines established from different patients were used for the study: ChaMel41 with HLA-A1, -A11, -B35, -B57, and Cw6; ChaMel84 with HLA-A2, -A11, -B35, -B60 (B4001), Cw3, and Cw4. The cells were grown in Nunclon™ triple flasks (Nunc, Wiesbaden, Germany) in DMEM supplemented with 5% fetal calf serum (FCS) and 5% newborn calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µM β-mercaptoethanol at 37 °C under 5% CO2. The cells were harvested by 10 min centrifugation at 800 × g, shock-frozen in liquid nitrogen, and stored as pellets at −80 °C. 2.5–10 × 10^6^ cells were used per cell line.

*Isolation of Peptides from MHC Class I Molecules*—MHC I class molecules were isolated as described in detail elsewhere (2, 3). Briefly, cells were lysed in lysis buffer (20 mM Tris-HCl buffer, pH 7.4, containing 0.3% CHAPS, 0.2% Nonidet P-40, 145 mM NaCl, 1 mM EDTA, 1 mM Pefabloc). Cell debris was removed by ultracentrifugation for 1 h at 100,000 × g. The supernatants were precleared by passage through an affinity column with a monoclonal antibody of irrelevant specificity and loaded onto the column with the monoclonal anti-human HLA-I antibody W6/32. Both antibodies were coupled to activated CH Sepharose (Amersham Biosciences) according to the protocol provided by the manufacturer. After absorption of the proteins, the column was washed with 20 mM Tris, 145 mM Tris-HCl, 145 mM NaCl, pH 7.4 (TBS), second with TBS with 0.3% CHAPS, third with TBS, fourth with TBS with 0.3% β-oc- tylglycoside, followed by TBS and ultrapure water. HLA-peptide complexes were eluted from the column with 0.7% TFA in ultrapure water and adjusted to 5% acetonitrile with 0.1% TFA. Peptides were separated from high molecular weight components by centrifugal ultrafiltration with a 3 kDa molecular mass cut-off (Centricon, Millipore, Schwalbach, Germany).

**Two-dimensional HPLC**—The filtrates were subjected to two-dimensional HPLC. The first dimension micro-HPLC was done with a Smart HPLC system (Amersham Biosciences, Freiburg, Germany) using a µRPC SC 2.1/10, C2/C18 reversed phase column (Amersham Biosciences) and an acetonitrile gradient of 5–95% of B in 90 min. (solvent A, 0.1% TFA in ultrapure water; solvent B, 90% of acetonitrile, 0.1% TFA). The second dimension nano-HPLC for subsequent MALDI-TOF-MS was performed with a solvent delivery system (Applied Biosystems Inc., Darmstadt, Germany) equipped with Acurate™ microflow processor (LC Packings, Amsterdam Netherlands) for flow splitting and a 5-µl sample loop. 20–40 µl of the first dimension HPLC fractions were lyophilized to near dryness, diluted with 5% acetonitrile, 0.1 TFA to 5 µl, sonicated in an ultrasonic bath, and applied at 300 nl/min onto a PepMap nano-HPLC column (75 µm, inner diameter × 15 cm) packed with 3 µm C18 stationary phase (LC Packings). Eluant A was 0.1% TFA in water/acetonitrile (95:5, v/v), eluant B 0.1% TFA in water/acetonitrile (10:90, v/v). The column was equilibrated with eluant A. After sample loading and washing with eluant A, the peptides were eluted with a linear gradient of 0 to 80% eluant B within 30 min at a flow rate of 200 nl/min. Fractions of 30 s were collected directly onto 600/384 TM AnchorChip MALDI targets (Bruker Daltonics, Bremen, Germany) using Probot fraction collector (Bioanalytische Instrumente, Bensheim, Germany). For ESI-MS/MS, an online Ultimate 3000 nanoHPLC system (Dionex, Darmstadt, Germany) was used. 40-µl first dimension HPLC fractions were prepared by drying in a SpeedVac and redissolving peptides in 5 µl of 5% acetonitrile, 0.1 TFA. Samples were loaded onto a C18 precolumn at a flow rate of 20 µl/min (2% acetonitrile, 0.05% TFA) for 10 min. Peptides were separated on a PepMap C18 nano-HPLC column (75 µm, inner diameter × 15 cm) at a flow rate of 220 nl/min with the following gradient: 5–60% of B over 60 min, followed by 60–90% of B over 5 min, and finally 90% of B for 5 min (eluent A, 0.1% formic acid in water; eluent B, 0.1% formic acid in acetonitrile).

**Mass Spectrometry**—For MALDI-TOF-MS, α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics) was used as matrix dissolved in 4 mg/ml in ethanol acetonitrile 0.1% trifluoroacetic acid (6:3:1). For increased peptide detection sensitivity and reduced matrix cluster formation, the matrix solution was supplemented with monoammonium phosphate (Sigma-Aldrich, Steinheim, Germany) and sonicated in an ultrasonic bath for 5 min. The matrix solution was dispensed onto an MTP AnchorChip™ MTP 600/384 TF target (Bruker Daltonics) with a GEloader pipette tip. Fractions collected from nanoHPLC were analyzed with a Reflex IV MALDI-TOF mass spectrometer (Bruker Daltonics) operating under Flex Control™ software (Bruker Daltonics). MALDI-TOF spectra were acquired in the positive ion reflector mode recording ions of 500 to 3,000 Da. Calibration was done externally with the peptide standard calibration II mixture (Bruker Daltonics). For MALDI-TOF post-source decay, the 29 kV reflector voltage was reduced stepwise to acquire the fragment spectra. Laser energy and number of shots per segment spectrum were adjusted manually. Analysis and annotation of both MS and post-source decay spectra for sequence analyses were done with FlexAnalysis (version 2.0, Bruker Daltonics). Annotation
of simple MS spectra was done automatically using the method provided by the manufacturer. Peaks with intensity five times higher than background in the 800–2000 m/z range were analyzed. ACTH 1–18 calibration file was used for internal post-source decay segment spectra calibration. Peptide sequences were computed from the fragment spectra with the Sequit! de novo sequencing software (4) and Mascot online search engine (Matrix Science, London, UK), both with a mass tolerance of 0.1 Da for the precursor ions and 0.5 Da for the fragment masses and oxidation on methionine as variable modification. The outcomes of both were blasted against NCBI database (version 20110627) for human entries (191,177).

ESI-MS/MS was performed with a MicrOTOF-Q (Bruker Daltonics) electrospray-ionization quadrupole TOF mass spectrometer in data-dependent mode: one MS spectrum followed by fragmentation of the five most intensive signals with optimized collision energy for fragmentation of HLA peptides and with argon as collision gas. Dynamic exclusion time of 1 min was set to avoid repeated fragmentation of the most abundant precursors. The MS and MS/MS spectra were processed using DataAnalysis (version 3.4) and Biotools (version 3.1) software (Bruker Daltonics). MS/MS searches for peptide identification were done on a local Mascot server (version 2.2) with a precursor mass tolerance of 200 ppm and a fragment mass tolerance of 0.2 Da, with no enzyme specified and oxidation of methionine and deamidation of asparagine and glutamine as variable modifications. The searches were done in Swissprot data bank (version 56.3) for human proteins (20,408 entries). Candidate sequences were controlled by manual inspection and independent searches including sequence tags. For validation, synthetic peptides were custom-synthesized by EMC microcollections GmbH (Tübingen, Germany).

For de novo sequencing by Sequit! MS/MS spectra were converted to mgf (mascot generic file) format generating monoisotopic mass lists for multiply charged fragments using DataAnalysis (version 3.4). For sequence computations, mass tolerance for both parent mass and fragment masses were set to 0.2 Da. The MS/MS spectra were inspected manually for specific neutral losses (–64 Da), indicating fragments containing oxidized methionine. When positive, oxidized methionine was included as optional amino acid for sequence computation by Sequit! The resulting sequences were blasted against the NCBI database (version 20090417).

Proteome and HLA Peptidome Data Analysis—All information (gene ID, gene symbol, protein sequence) for proteomic data analyses was obtained from the NCBI database. Protein classifications according to biological function or subcellular localizations were done using data of the manually curated human protein reference database (5). HLA ligand classifications according canonical HLA binding motifs were predicted with SIFPEITHY (6).

Transcriptome Analysis—RNA expression was determined with the Illumina human whole genome expression profiling system WG-6 (ServiceXS, Leiden, Netherlands) with mRNA and cDNA prepared from the ChaMel47, -84, and -105 melanoma cell lines and normal human melanocytes at the MPI for Molecular Genetics (Berlin, Germany). mRNA expression data analysis was done with the Illumina BeadStudio software with data normalization through an average normalization algorithm. The arbitrary signal values for 48,803 transcripts ranged up to 36,819; 60 was set as the background cut-off value.

Data Analysis—mRNA expression and turnover data of HLA peptide source proteins were compared with entire transcriptome data and proteomic data of 4,106 proteins published by Cambridge et al. (7). The values were normalized for the total and the HLA peptide source protein populations, expressed as bar diagrams and cumulative population plots processed by two-sample Kolmogorov-Smirnov statistics with Origin (version 8.6). Differences with \( p \leq 0.05 \) were considered as significant.

T Cell Assays—The immunological activity of epitopes identified from HLA peptidomes was tested with peripheral blood mononuclear cells (PBMC) or tumor-infiltrating lymphocytes from melanoma patients and PBMCs of healthy control donors by ELISpot assays as described detail elsewhere (8). Briefly, PBMCs were isolated from peripheral blood of melanoma patients and healthy donors by Ficoll (Pharmacia, Heidelberg, Germany) density gradient centrifugation and used fresh or cryopreserved in FCS with 10% dimethyl sulfoxide at −140 °C. Tumor-infiltrating lymphocytes were isolated from melanoma metastases by removing tumor and tissue cells including fibroblasts. T lymphocytes were stimulated in cell culture with Dynabeads CD3CD28 (Invitrogen), expanded in IL-2 (100 units/ml) containing medium and cryopreserved as described above. For the assays, the cells were pulsed with the synthetic peptide (10 \( \mu \)g/ml) in Ex Vivo 15 serum-free medium (Biowhittaker) in ELISpot assay plates (MultiScreen, Millipore, Schwalbach, Germany) coated with anti-human INF-γ (Endogene, Pierce Biotechnology, Inc.) using phytohemagglutinin (3 \( \mu \)g/ml) (Sigma-Aldrich) as positive and medium as negative controls. After 16–20 h at 37 °C, the cells were washed off, and IFN-γ was captured on the plates counterstained with a matching biotinylated anti-human IFN-γ antibody (Endogene, Pierce Biotechnology) and, subsequently, alkaline phosphatase-labeled streptavidin (Roche Diagnostics). Positive spots were visualized through 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt/nitrotetrazolium chloride (Moss, Inc., Pasadena, CA) dye reaction and recorded and analyzed with a Bioreader 3000 (BioSys, Karben, Germany).

RESULTS

HLA Peptidomes of Melanoma Cell Lines—HLA I-bound peptides of four human melanoma cell lines (ChaMel41, ChaMel84, ChaMel100, ChaMel105) established from metastases of different patients were extracted from immune affinity purified HLA-I and isolated by reversed-phase micro-HPLC followed by nano-HPLC. The tumor cells had similar morphology, growth patterns, and generation times, and the patients had been HLA-typed (supplemental Table S1). All cell lines shared HLA-A11, three B35, two B51, and three Cw4. Based on published sequence motifs for HLA epitopes and allowed amino acids in the anchor positions as evidenced by epitopes identified from the respective HLA, extended sequence motifs for peptide binding by the HLA can be defined. Taking these motifs, different HLA may bind peptides with the same or very similar sequence motifs and four or five of the six
expressed HLA of the cell lines in any comparison of two, three, and four cell lines had identical or compatible peptide binding requirements.

The peptides were analyzed by MALDI-TOF or electrospray ionization quadrupole TOF MS and compared according to their HPLC retention times and masses to identify identical peptides in the peptidomes of the different cell lines (see Fig. 1 for an example). To align the different HPLC runs, 19 peptides that had been sequenced (see below) and found in all of the peptidomes were used as standards. In all, 3,203 peptides were detected: 827 for ChaMel41, 1,054 for ChaMel84, 557 for ChaMel100, and 765 for ChaMel105 (supplemental Fig. S1A).

FIGURE 1. Mass spectra of peptides isolated from the HLA of melanoma cell line ChaMel100 (A), ChaMel41 (B), ChaMel84 (C), and ChaMel105 (D). The peptides were identified by their retention times in microHPLC, and masses were determined by MALDI-TOF-MS in reflector mode. The peptide masses indicated with asterisks were found in all four peptidomes, sequenced, and used to calibrate the HPLC runs.
lines. Considering the number of HLA with identical or shared peptide motif requirements, an upper number of potentially shared peptides can be calculated (supplemental Table S1). Of these, between 6 and 18% of the peptides were found to be shared by two cell lines, between 5 and 13% by three cell lines, and 10% by all four cell lines. Thus, only a small fraction of the expected common peptides are actually shared, indicating a high degree of individualization of the peptidomes of different melanoma cell lines and, by extension, tumors. This high degree of individualization was confirmed by comparison of the MS/MS fragmentation spectra of 451 peptides of the cell lines ChaMel41, 84 and 100 of which only 12 (4% of the expected) were found to be shared by all cell lines compared (data not shown).

**Identification of HLA-bound Peptides**—Peptide sequences were computed from MALDI-post-source decay and electrospray ionization quadrupole TOF MS/MS spectra through two independent processes, database-dependent Mascot searches, and database-independent de novo sequencing using the Sekuit! software and BLAST searches so that mutations and modifications could be identified. The sequences of 499 peptides were resolved, with 192 by MALDI-TOF MS and 395 by electrospray-ionization quadrupole TOF MS (supplemental Table S2 and supplemental Fig. S2); of these, only 88 were identified by both technologies. The two ionization technologies thus provide largely complementary information on peptide sequences, which may relate to the known differences in the mechanisms of peptide disintegration. In cases of Mascot scores below 35 or divergent results of Mascot and Sekuit! analyses, the sequences were validated with synthetic peptides, in all 106 sequences. Of the 499 sequenced peptides, 375 were non-redundant peptides from 306 different proteins. Nineteen peptides were found in the peptidomes of all four tumor cell lines, 14 in those of three cell lines, 39 in two (supplemental Fig. S1B), and 303 peptides only in one of the tumor cell HLA peptidomes. Nine of the 375 peptides were identified by comparing retention times and the fragmentation patterns with spectra of natural and synthetic peptides. In all cases, the results of the MASCOT searches matched the de novo sequencing results, meaning, no mutated peptides were found in comparison with the database entries.

Eighty six of the 375 non-redundant peptides had been identified in earlier HLA peptidome studies (supplemental Table S2). Five HLA epitopes that had previously been predicted according to sequence motifs or determined by sequence scans with synthetic peptide were confirmed by our analysis as naturally presented. Of these, GTATLRLVK of gp100 was previously predicted as HLA-A3, and HLA-A11 restricted melanoma-associated antigen and induces T cell responses in patients (9). SAYGEPRKL of MAGE-A1 had been identified by reductive cloning of the MAGE A1 cDNA and sequence scans (10). NLKLKHLHF of the retinoblastoma binding protein 4, GVMPSHFSR of the ribosomal protein S19, and ASFDKAKILK of thymosin β 10 had been predicted by their sequence motifs (11–13). The remaining 289 epitopes had hitherto not been described.

For 41 source proteins, several HLA ligands were identified, with some presented by different HLA (supplemental Table S3). One epitope derived from the G-protein pathway suppressor GPS-2 occurred in three different forms with non-, mono-, and dimethylated arginine as described in detail earlier (14). In 16 cases, core sequences were identified with different C- and/or N-terminal extensions. These differently extended sequences will give rise to different antigenic structures and may be counted as truly different epitopes. Some of these variants are shared among cell lines, but a large number were discriminately detected despite shared HLA. Particularly in ChaMel41, and to a lesser extent in ChaMel100, variants of the same core sequence were found. The occurrence of such different sequence variants in different cell lines suggests differences in antigen processing, e.g. N-terminal variations, in addition to possible proteasomal effects, could indicate different actions of aminopeptidases.

**Classification of the Source Proteins of HLA Ligands by Subcellular Localization and Biological Functions**—The source proteins of the identified HLA epitopes were classified by their subcellular localization using the manually curated human protein reference database (5). The largest fraction (129 proteins) of the source proteins was nuclear proteins (Fig. 2A); 128 pro-
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FIGURE 3. Comparison of the mRNA expression levels of the source proteins of the identified HLA ligands with the total transcriptome of melanoma cell lines. The mRNA expression levels for the melanoma cell lines were averages of three cell lines ChaMel47, -84, and -100, detected and quantified with an Illumina WG-6 Chip as described under “Experimental Procedures.” The total number of transcripts (48,803) and the 535 transcripts of source proteins were clustered into 52 groups according expression values, normalized and displayed for direct comparison. The cumulative population plot is displayed as the inset. The cumulative distribution distance was calculated as $D = 0.61 (p = 1.61 \times 10^{-7})$.

Proteins were from the cytoplasm, and the remaining were exported, including outer membrane proteins. Some of the proteins are present in different compartments. Proteins of every major subcellular compartment were identified as source of HLA ligands. Also, the classification of the source proteins by biological functions revealed a broad representation of the various cellular functions. Proteins involved in cell growth and/or maintenance (22 proteins), cellular metabolism and energy pathways (26 proteins), protein metabolism and translational elongation (37 proteins), transport (27 proteins), proteins with cell type-specific regulatory functions involved in cell communication and signal transduction (61 proteins), regulation of transcription (31 proteins), RNA processing (21 proteins), cell cycle control (four proteins), DNA binding (six proteins), DNA replication (two proteins), immune response (four proteins), apoptosis (four proteins), and such with unknown functions (35 proteins) were found (Fig. 2B). Twenty source proteins that are involved in processes such as angiogenesis, cell proliferation, or tumor suppressor functions could be of pathological relevance (supplemental Table S4). Structural proteins and metabolic enzymes appeared underrepresented compared with their share in the overall amount of cellular proteins (supplemental Table S2).

mRNA Expression of Identified HLA Ligands—We investigated the peptidome and transcriptome data of the melanoma cells with respect to the hypotheses that the HLA peptidomes represent preferentially proteins with high expression levels or overexpressed proteins compared with normal cells of the same histotype. Although protein expression data might be the better basis for such analyses, the lack of suitable quantitative proteome data allowed us, similar to others, to resort to using transcriptome data. For transcriptomics, we used whole genome array data for 48,803 transcripts (Illumina Chip) for the cell lines ChaMel84 and ChaMel105 plus the independent cell line ChaMel47 and compared the distribution of the expression levels of the average of all transcripts with those of the source proteins of the identified HLA ligands. The expression levels are expressed as arbitrary units with a maximum at 36,819. For statistical analysis the transcripts in these two populations were divided according to expression levels into 52 groups and displayed as normalized histogram and cumulative population plot (Fig. 3). The data were then processed by Kolmogorov-Smirnov analysis to calculate the maximal distance (D) between the two populations and statistical significance ($p$ value). Although there is a tendency that source proteins of HLA ligands are expressed at slightly higher levels, 11% of their transcripts in our analyses were $>10,000$ arbitrary units versus $1.1%$ for the entire transcriptome; there is no preference for source proteins with high expression levels. The results were similar when the cell lines ChaMel84 and ChaMel105 for which we had matching transcriptome and peptidome data were analyzed separately: their fraction of source proteins with transcript levels above $10,000$ arbitrary units was $11$ and $14%$, respectively (supplemental Fig. S3).

To assess the degree of overexpression, we calculated the ratios of the expression levels for each transcript in the melanoma cells versus the melanocytes and took a ratio of 5 as cutoff for overexpression (Fig. 4A). For the average of the three melanoma cell lines, only five proteins were overexpressed: annexin A1, MAGE 1, lysyloxidase homolog 3, matrix Gla protein, and the nerve growth factor-inducible vascular endothelial growth factor (VGF) (Fig. 4B). In the corresponding separate analysis for the two melanoma cell lines ChaMel84 and -105, two source proteins were identified as overexpressed in ChaMel84, lysyloxidase homolog 3 and annexin A1, and four in ChaMel105, THO complex 4 protein, a membrane-associated transport protein, nucleopore complex protein NUP205, and matrix Gla protein (supplemental Fig. S4).

Turnover of HLA Peptidome Source Proteins—It is often stated that the HLA peptidome would represent the protein degradome, i.e. the degraded proteins, rather than the cellular proteome. This would imply a correlation of the presentation...
and the turnover rates of the source proteins of HLA epitopes. For classification of HLA peptide source proteins according to their turnover rates ($k_{\text{deg}}$), we used published proteomic data for the turnover rates of 4,106 proteins in non-dividing HeLa cells (7). This set of data included the turnover rates for 184 of the 306 source proteins of our study, and we compared their turnover rates with the entire set of 4,106 proteins. For this comparison, the proteins were categorized according to their turnover rates and the normalized frequencies plotted as a histogram and cumulative population plot (Fig. 5). This comparison showed that the distribution for both populations, HLA peptide source proteins and all 4,106 proteins for which $k_{\text{deg}}$ values had been published superimpose, suggesting that the HLA peptidome reflects the turnover of the cellular proteins. The half-life times of the source proteins range between 8.8 and 138 h with a peak $\approx 46$ h and can be classified as slow to medium range turnover proteins. Because no turnover data were reported for the remaining source proteins, we can conclude that at least 60% of the identified HLA peptide source proteins fall into this range.

**HLA Specificity of the Identified Epitopes**—About 65.9% of the identified HLA ligands were nonapeptides; octapeptides, decapeptides, and undecapeptides were present with 6.4, 16.8, and 8.8%, respectively (Fig. 6). Exceptionally, heptapeptides, dodecapeptides and tridecapeptides were found. The identified epitopes thus display the typical size range of HLA I ligands. To assign the epitopes to the HLA alleles to which they likely bind, we used the HLA allele-specific peptide binding motifs listed in the SYFPEITHI database for the HLA of the four melanoma cell lines (supplemental Table S1). One hundred sixty five non-redundant peptides (43.7%) matched the HLA-A11 binding motif with a basic amino acid at the C terminus (Fig. 6). Seventy three peptides (19.3%) fit the HLA-A24 binding motif with the predominant aromatic amino acid tyrosine at the second position and aromatic or large hydrophobic amino acid at C terminus of peptides. Forty five peptides (11.9%) could be classified as HLA-A01, 32 (8.5%) peptides could be classified as HLA-B35, 21 (5.6%) peptides could be classified as HLA-B-51, and 18 (4.8%) peptides could be classified as HLA-A02 epitopes. Some of the HLA-B35 ligands do not have the canonical proline, valine, or alanine at position 2 but instead have serine, arginine, leucine, or tyrosine as reported for some HLA-B35 ligands, which indicates a greater tolerance to amino acid variation in this position than that represented by the binding motif. Four ligands were assigned to HLA-A32 based on the sequence of a T cell epitope published as a HLA-A32 ligand previously (15). SYFPEITHI lists no binding motif for this HLA. Instead, the assignment was done by the classification of HLA-32 as member of the HLA-A01 supertype that displays the same peptide binding requirements (16). Six HLA ligands were classified as HLA-C binders. Two of the ligands, NYIDKVRFL and YIDKVRFL, were identified in ChaMel41 and ChaMel84, and through classification by the respective binding motifs were identified as HLA-A24 and HLA-Cw4 ligands. Twelve epitopes could not be assigned to any HLA binding motifs. Two epitopes, SAYGEPRKL and NLKLKHLSF, had previously been published as HLA-Cw3, HLA-Cw16, or HLA-B08 binders, respectively (10, 11). However, the melanoma cells from which the peptides had been eluted do not express these HLA. These two peptides must therefore bind to other HLA. In addition, two ligands with the
characteristic binding motifs for HLA-B08 were found and are included in supplemental Table S2. In all, 287 peptides were assigned as HLA-A11 ligands, 77 for ChaMel41, 82 for ChaMel84, 91 for ChaMel100, and 37 for ChaMel105 (supplemental Fig. S5). Nineteen of these peptides were shared by all four cell lines, indicating a moderate degree of heterogenicity (51% of expected).

Immunogenicity of HLA Ligands—To test the immunogenicity of the identified HLA ligands, tumor-infiltrating lymphocytes from the melanoma metastasis from which the tumor cell line ChaMel84 had been established were tested ex vivo for responses to 96 peptides randomly selected from the entire set of identified HLA ligands. The reduction in numbers for the test was necessary because of limitations with the biological materials. The tests were ELISpot assays for interferon γ, and a stimulation index of two was taken as cut-off for response. 21 of the 96 peptides (22%) induced T cell responses (Table 1), indicating a broad antigenicity of the tumor cells in the patients as well as the capacity of the T cells in the tumors to mount immune reactions. The active peptides are likely presented by HLA-A11, -A24, -B35, -B51, and -B57 with a prominence of HLA-A11-restricted responses. Among the targets of the T cells are a number of proteins with potential pathological relevance for tumor development, including proteins of tumor suppressor and signaling pathways, proteins involved in cell cycle control, gene regulation, protein metabolism, transport, and intercellular communication (Table 1, supplemental Table S2).
TABLE 1

Responses of tumor-infiltrating CD8 T cells to identified HLA ligands

In addition to the listed peptides, the peptides AAYNRYFRK (matrix Gla protein), SASYEPKRL (MAGE A1), LPHYGAETFR (lysozyme homolog 3), ALLDRIVSV, LLIGHLerv and SLSYIIKl (NUP205), GTADVHEFR (THO C4), and LLTGEQFQV (membrane-associated transporter), all overexpressed source proteins, had been tested as well but did not stimulate any T cell responses (SI < 1.5). SI, stimulation index.

| Peptide | Protein | HLA     | SI   |
|---------|---------|---------|------|
| ATYSSSHYHK | SEC31-like 1 | A11 2   |
| AYQGDFLARFK | PRELI | A11 2   |
| SIFGDRVVK | TMCO1 | A11 2   |
| GSDDVIHR | PHRF1 | A11 5   |
| RTWNPTGK | RINT1 | A11 2.25 |
| SVSFVHRV | hnRNP L | A11 3.75 |
| ATIDLTK | Annexin A1 | A11 3   |
| STMHTPGMNR | FOXO1 | A11 5.75 |
| SSSLFFRK | Retinoblastoma-like2 (p130) | A11 2.5 |
| SISFGDFK | Liprin-β1 | A11 3   |
| GTSSSISVR | F-box and WD-40 domain protein 1B | A11 2.25 |
| RVTYPAKK | Ribophorin II | A11 2.25 |
| RTYDREGFKK | Nudix motif 4 | A11 3   |
| VYLDKBFRL | Guanine nucleotide binding protein-like 3 | A24 2.4 |
| RFEERQHAYF | Ccn, crooked neck-like 1 | A24 2.5 |
| RYQWQMERF | eIF3 homolog 2 | B24 6.25 |
| YPKPPKVK | Ubiquitin-conjugating enzyme E2E3 | B51 3.75 |
| SSVPGVRLL | Vimentin | B35 2.5 |
| NLRKLHSF | Retinoblastoma binding protein 4 | B57 3.75 |
| KARILSSAALF | GVE nerve growth factor inducible | B57 3.75 |
| GRAPQVVL | DEAD box helicase 21 | B35 2   |

In addition, 29 randomly selected HLA-A11-restricted peptides were tested as described previously with T cells from PBMC of eight unrelated melanoma patients and four healthy donors, all HLA-A11 (Table 2). 22 of the 29 peptides (72%) did induce T cell responses in the patients. All patients tested had reactive T cells in their peripheral blood for between 2 and 15 of the 29 epitopes. With PBMC of the healthy donors, T cell responses against 11 peptides (38%) were detected with three of the four donors responding to two and one to six peptides. The potential pathological significance of the source proteins of these epitopes was similarly ranged as those for the tumor-infiltrating lymphocytes. The assays for both compartments, tumor and peripheral blood, and for patients whose tumor cells had been used to identify the epitopes as well as unrelated patients, demonstrate a broad immunogenicity of the tumor cell HLA peptidomes and a broad range of tumor-specific T cell responses in the patients. Healthy donors responded to fewer epitopes and on average less strongly. Among the tested peptides were also the epitopes derived from the eight overexpressed source proteins. Only the epitopes of two of these proteins, annexin A1 and VGF, did induce T cell responses (Table 1); the epitopes of the other six proteins were negative.

DISCUSSION

Four key conclusions are evident from the analysis of the HLA peptidomes of tumor cells reported herein. First, the peptidomes are heterogeneous and individualized even regarding shared HLA. Second, they are highly immunogenic when tested across different individuals. Third, the HLA-bound peptides are derived from proteins of average expression levels that only exceptionally are overexpressed compared with normal cells. Fourth, the majority of the peptides are from proteins with short to medium range turnover rates. Although these were investigated in the context of the present report with respect to tumor immunology, these conclusions may be valid beyond. This would imply that the MHC-dependent mode of antigen recog-
as melanoma-associated antigen in experiments with T cells from melanoma patients (25). The enzyme lysyl oxidase homolog 3 (LOXL3) belongs to the copper-dependent amino oxidases family and is responsible for cross-linking collagen or elastin. Overexpression of LOXL3 was detected in highly invasive melanoma and breast carcinoma cells (26). It may thus play a role in tumor progression and metastasis and could be an interesting target for immunotherapy. The other five ANXA1, matrix gla protein, VGF, NUP205, and THO C4 had previously not been described in the context of melanoma. ANXA1 was published as a putative proinvasive marker for lung adenocarcinoma (27). Overexpression of matrix gla protein in glioblastoma was shown to increase metastatic capacity of the tumor cells and is associated with a worse outcome (28). VGF is normally expressed in nerve cells and was proposed as a marker for pulmonary neuroendocrine tumors (29). Finally, THO complex 4 and the nuclear pore protein Nup205 both possess transporter activity but were hitherto not described as tumor-related.

In our studies, HLA ligands derived from ANXA1 and VGF were recognized by tumor-infiltrating T cells of melanoma patients and thus might be additional targets for immune therapy. The majority of the epitopes from overexpressed proteins did not induce T cell responses detectable in tumor-infiltrating lymphocytes or peripheral blood. In conclusion, structural deviations from normal proteins such as mutations, modifications, or overexpression may in exceptional cases translate into immunogenicity but are not prerequisite for tumor immunogenicity. Why normal cellular proteins dominate tumor antigenicity remains unresolved but may be related to immune-regulatory processes in the tumors and is being investigated.

The data substantiate the notion that the HLA-dependent mode of antigen recognition enables the immune system to scan all, including internal, cellular compartments for potentially pathological changes. This is probably best illustrated by the finding that in the present study 42% of the identified source proteins for the HLA ligands are protein of the nucleus, the compartment where cell cycle control, DNA replication, gene regulation, and tumor suppressor action may determine cell behavior and fate. This percentage is comparable with the 43% nuclear proteins reported by Hickmann et al. (30) for peptides presented by HLA-B1801, which has no requirement for basic anchor amino acids. This notwithstanding, the high representation of nuclear proteins, normally enriched in basic amino acids necessary for protein translocation into the nucleus and interaction with DNA could also be explained by the preferences of HLA-A11 peptides with basic amino acids at C terminus and a high efficiency of mass spectrometric detection of such peptides.

Detailed knowledge of HLA peptidomes can lead to a better understanding of the peptide specificities of the various HLA, their source proteins, antigen processing, and the basis for identification of pathological developments by the immune system. Additionally, such data are basis for the development of more refined prediction algorithms for potential HLA ligands that take into account the overlap of the specificities of the peptidomes of different HLA and can cope better with the length variations of HLA ligands than existing algorithms.

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