

Isolation of Novel HLA-DR Restricted Potential Tumor-associated Antigens from the Melanoma Cell Line FM3¹

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ABSTRACT

Endogenous peptides bound to the constitutively expressed MHC class II molecules HLA-DR and HLA-DQ of the melanoma cell line FM3 were examined. By a combination of analytical methods (narrow bore and capillary reversed-phase high-performance liquid chromatography with subsequent spotting on polyvinylidene difluoride membranes, matrix-assisted laser desorption ionization mass spectrometry, and Edman microsequencing), we were able to isolate and identify a panel of HLA-DR4/2 (HLA-DRB1*0401/0201/DRB5*0101)-associated self-peptides from the melanoma cell line FM3. Among ubiquitously HLA-DR-associated peptides such as peptides from the class II-associated invariant chain peptide region of the invariant chain, HLA-class I, the transferrin receptor, and the IFN- γ receptor, we identified several potential tumor-associated antigens stemming from the MHC class I-restricted tumor antigen gp100, the Ca²⁺-binding protein annexin II, and proteins from the hsp70 family. Chinese hamster ovary cells cotransfected with *HLA-DRA*, *DRB1*0401*, and *CD80* genes were shown specifically to prime T lymphocytes from HLA-DRB1*0401 donors to the annexin II and gp100 peptides. These results demonstrate that MHC class II molecules expressed by melanoma cells potentially present a variety of novel antigens to the immune system, some of which could be exploited for immunotherapy.

INTRODUCTION

It is now firmly established that many different types of tumors are immunogenic for T cells and that specific immune responses can result in tumor destruction. Active immunotherapy relies upon triggering antitumor immune responses *in vivo* by immunizing the patient with tumor cells with or without transferred genes or with autologous APCs³ pulsed with tumor cell products such as synthetic peptides representing tumor-specific epitopes. Adoptive immunotherapy, on the other hand, does not rely on the presence of an intact host immune system but on the transfer of presensitized effector cells or antitumor molecules into the patient. MHC class I-restricted CTLs can recognize and destroy tumor cells *in vitro* and *in vivo* but require CD4⁺ MHC class II-restricted help for optimal responses. Most published work has thus far concentrated on CTLs, although it is known that CD4⁺ T cells may be equally or more important in tumor rejection, as shown in several animal models (1).

Thus far, a limited number of HLA class I-restricted tumor antigens such as MAGE-1 and -3, BAGE, GAGE, MART-1/Melan-A, gp100 (Pmel17), gp75 (tyrosinase-related protein TRP1), and tyrosinase have been identified in melanoma cells (2). Furthermore, class I-restricted cytotoxic T cells specific for these tumor peptides have been found by several groups, suggesting that they do represent antigens in

the T-cell repertoire (3–8). Several peptide epitopes of the target antigens have been identified recently (9–20). Currently, there is very little known regarding potential tumor antigen peptides in the context of MHC class II. Recently, however, two HLA-DRB1*0401-restricted tyrosinase epitopes have been described (21). These two epitopes, Ty 56-70 and Ty 448-462, showed intermediate and weak MHC binding affinities. The authors also examined single amino acid derivatives of these two epitopes for their binding affinity to HLA-DRB1*0401 and the corresponding T-cell stimulation capacity. Certain modified peptides were found to bind and stimulate better than the wild-type peptides. Thus, in human melanoma, it is interesting that one of the several targets recognized by CTLs in a class I-restricted manner (22) is apparently also recognized by CD4⁺ cells in a class II-restricted manner (21, 23). Another group has reported HLA-DQ- as well as DR-restricted recognition of melanoma antigens shared between different melanomas, but the identity of the peptides recognized was not determined (24). These reports suggest that selecting peptides recognized by both helper T cells and CTLs might be beneficial in vaccinating against cancer.

To establish an *in vitro* model for antimelanoma responses, we took advantage of the FM3 melanoma cell line, derived from an elderly patient, which is highly immunogenic (25) and expresses MHC class II as well as MHC class I molecules. Furthermore, it expresses the melanoma-associated antigens HMB-45, tyrosinase, MART-1/Melan-A, MAGE-3, and gp100 (Pmel17). It was able to act as an APC for both class I and II-restricted primary responses (26). To initiate studies on the nature of the class II-restricted antigens recognized by T cells on the surface of FM3, we undertook the molecular characterization of endogenous peptides eluted from the HLA-DR molecules expressed by these melanoma cells.

MATERIALS AND METHODS

Cell Lines and Antibodies. The melanoma cell line FM3 (HLA-DR2 (DRB1*02x), DR4 (DRB1*0401), DQ1 subplit DQ6 (DQB1*0602), DQ3 subplit DQ7 (DQB1*0301), HLA-A2 (A2.1), 3, HLA-B7, 44 was established from a metastatic tumor of a 70-year-old female. FM3 was shown to be highly immunogenic in mixed lymphocyte tumor culture assay (25). EBV-transformed B-LCLs LD2B (HLA-DRB1*0201/DRB5*0101), BSM (HLA-DRB1*0401/DRB4*0101), WT100BIS (HLA-DRB1*0101), COX (HLA-DRB1*0301/DRB3*0101), and BM21 (HLA-DRB1*1101/DRB3*0202) served as sources for the HLA-DR alleles. The expression of the HLA-DRB3 and DRB4 alleles is low compared to that of the DRB1 alleles (<10%). Cells were cultured in RPMI 1640 (Life Technologies, Inc., Eggenstein, Germany) supplemented with 5% FCS (Life Technologies, Inc.), 20 mM HEPES, 2 mM glutamine, and antibiotics and expanded in roller bottles to 1.0×10^{10} cells (FM3) and $1-2 \times 10^9$ cells (LD2B, BSM, WT100BIS, COX, and BM21). The mAb L243, which recognizes a nonpolymorphic determinant present on HLA-DR molecules, was obtained from the American Type Culture Collection. mAb Tü22, which binds a complex epitope on HLA-DQ dimers, was kindly provided by C. A. Müller (Tübingen).

HLA-DR Purification. HLA-DR molecules from FM3 cells were purified by means of affinity chromatography using L243-coupled CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden). The cell lysate containing 2% (w/w) NP40 was cycled overnight through a precolumn (CNBr-activated Sepharose

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³ The abbreviations used are: APC, antigen-presenting cell; TFA, trifluoroacetic acid; CHO, Chinese hamster ovary; RP, reversed-phase; HPLC, high-performance liquid chromatography; CLIP, class II-associated invariant chain peptide; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; B-LCL, B-cell lymphoblastoid cell line; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; HA, hemagglutinin.

saturated with Tris-glycin), three L243 columns, and one Tü22 column. After washing with 10 column volumes of washing buffer [50 mM sodium phosphate, 100 mM NaCl, and 0.1% Zwittergent 3-12 (Calbiochem, La Jolla, CA)], two alternative elution protocols were used. For isolating the self-peptide pool, two L243 columns were extensively washed with water to remove the detergent. The elution was performed using water and TFA (pH 2.0). Subsequently, peptides were separated from the HLA molecules by ultrafiltration using a 20-kDa cutoff membrane (Sartorius, Göttingen, Germany). For isolation of intact HLA-DR or HLA-DQ molecules with bound self-peptides, the elution was performed with a 100 mM sodium phosphate buffer containing 0.1% Zwittergent 3-12 (pH 11.0). The eluted protein solution was immediately neutralized with 1 M citric acid and stored at 4°C until use. The latter protocol was performed with the third L243 column and the Tü22 column.

SDS-PAGE Analysis of Class II Molecules. The intact class II peptide complexes (HLA-DR and DQ) as well as the acid-treated peptide-released DR molecules were checked for quantity and quality of the isolates by SDS-PAGE using the method of Laemmli (27). Quantification was performed by using a protein standard with known protein concentration (Pharmacia) and by comparing the protein concentration measured by the Bradford method, subtracting the impurities prone to unspecific adsorbance to the Sepharose, which consisted mainly of actin and albumin.

Peptide Pool Chromatography. The self-peptide pool was separated by RP-HPLC using a C18 1-mm column (250 MGL-SDSI-30/5; Scientific Glass Engineering, Weiterstadt, Germany). The gradient was 7-77% B (1%/min); A, 0.1% TFA; B, 0.08% TFA and 84% acetonitrile with a flow rate of 27 μ l/min. The absorbances at 215 and 295 nm were simultaneously detected (UV detector SPD 10A; LC-Packings). Peaks were collected manually and immediately frozen in liquid nitrogen.

Peptide Analysis. MALDI-MS (Finnigan MAT, La Jolla, CA) served to determine the masses and the homogeneity of the collected peaks. Homogeneous peaks were directly submitted to the Edman protein microsequencer (ABI 476A; Applied Biosystems, Weiterstadt, Germany). Heterogeneous samples were rechromatographed by a C18 180- μ m capillary column (LC-Packings) using separation buffers A [10 mM NH₄OAc (pH 6.0)] and B [10 mM NH₄OAc, 84% acetonitrile (pH 6.0)] with a flow rate of 1.5 μ l/min. The resulting peaks were spotted directly on a polyvinylidene difluoride-sequencing membrane (Immobilon-P; Millipore, Eschborn, Germany) using an automatic robot arm with a capillary (Probot; BAI, Benzheim, Germany), cut out, and sequenced. The sequencing data were compared with the Swissprot protein sequence database at the European Bioinformatics Institute (Cambridge, United Kingdom) to identify the sources of the peptides. The mass of the identified peptides determined by MALDI-MS was a very useful tool not only to estimate the length of a peptide but also to ascertain or exclude a databank match. This was especially important because a few amino acids are inherently difficult to detect in Edman sequencing (Cys, Trp, and Arg).

Peptide Synthesis. All peptides without methionine were synthesized in our lab on a multiple peptide synthesizer (model SMPS 350; Zinsser Analytik, Frankfurt, Germany). Peptides containing at least one methionine were synthesized with a Milligen 9050 Pepsynthesizer (Millipore), because this synthesizer allows peptide synthesis under nitrogen atmosphere to protect methionine from oxidation. We used the Fmoc/tBu strategy for both synthesizers and purified the crude peptides by RP-HPLC (Merck-Hitachi) to a purity of >90%. The sequences of the purified peptides were determined by mass spectrometry (model API III; Sciex, Toronto, Canada). Hemagglutinin 306-318, PKYVKQNTLKLAT, as the DR1, 2, and 4 binder (28); hsp65, 3-13, KTIA Y-DEEARR, as the DR3 binder (29); and hsc70 239-250, NHFIAEFKRKHK, as the DR11 binder (30) were amino-terminal fluorescently labeled with AMCA.⁴

Peptide Binding Assay. The peptide binding assay was performed as described previously (31, 32). Briefly, purified HLA-DR molecules (200 nM) were incubated for 40 h at 37°C with an AMCA-labeled peptide for HLA-DR1, 2, or 4 with influenza HA 306-318 (1.7 μ M) and coincubated with different concentrations of unlabeled peptides as competitors in binding buffer [150 mM sodium phosphate, 0.1% Zwittergent 3-12, 15% acetonitrile (pH 5.5)]. The HLA-peptide complexes were separated by high-performance size-exclusion chromatography using a Pharmacia Superdex 75 HR 5/20 column (Pharmacia) with a flow rate of 0.4 ml/min. UV detection of the protein at 214 nm and

detection of the protein-bound fluorescence at 350 and 450 nm was measured simultaneously using a Hitachi-Merck UV detector (L 4000) and a Hitachi-Merck fluorescence spectrophotometer (F 1050). The relative fluorescence of each sample was expressed as the ratio of protein-bound fluorescence signal and the corresponding UV signal. In competition assays, the decrease of the relative fluorescence compared to that of a control sample without a competitor peptide was calculated.

Cellular Assays. CHO K1 cells were transfected with human cDNAs encoding *HLA-DRB1*0401* and *HLA-DRA* and cotransfected with *CD80* in addition to these genes, as described previously (33). Each batch of CHO transfectants was cryopreserved, and one sample of each was checked for retention of expression of human gene products before that batch was released for use as APCs. CHO APCs were preincubated for 2 h at room temperature at pH 7.0 with annexin II 208-223 or gp100 44-59 synthetic peptides at 10 μ g/ml and then fixed for 2 min with 0.025% glutaraldehyde. Pulsed CHO cells (or unpulsed cells as a negative control) were then cocultured with PBMCs obtained from normal healthy donors carrying the *HLA-DRB*0401* allele and thus not able to respond to the DR antigens of the CHO cells by alloreactivity. Coculture was carried out in 16-mm-diameter Costar wells, using 1×10^6 PBMCs and 5×10^5 APCs per well in 2 ml of culture medium (RPMI 1640 supplemented with 10% heat-inactivated nontransfused human male serum). After 7 days, 1.5 ml of medium were removed and replaced by fresh medium containing 5×10^5 peptide-pulsed (or unpulsed) APCs to restimulate the responding cells. After a total of 14 days of culture, cells were removed and plated at 2×10^4 cells/well into round-bottomed microtiter plates to which the same number of CHO cells was added as stimulators. These APCs were either pulsed with the specific annexin or gp100 peptides or pulsed under the same conditions with the gp100 44-59 or annexin 208-223 peptides. Replicate plates were set up containing all cultures performed in triplicate. Each well received 37 kBq of tritiated thymidine (Amersham-Buchler; specific activity, 185 GBq/mmol) after 24, 48, or 72 h. Radioactivity incorporated into the nuclei of proliferating cells was isolated by harvesting onto glass fiber sheets and quantified by liquid scintillation counting.

RESULTS

HLA-DR Molecules Lose Their Native Conformation after Acid Peptide Release but not after Basic Elution. To show that constitutively expressed class II molecules on FM3 cells are in fact functional, we examined their behavior in SDS-PAGE. As can be seen in Fig. 1, the HLA-DR molecules eluted at pH 11 showed normal size and conformation in SDS-PAGE. The boiled samples generated two bands, corresponding to the α -chain at 36 kDa and the β -chain at 31 kDa. The same isolate not boiled showed a diffuse dimer band at about 60 kDa but still showed weak monomer bands. In contrast, the pH 2-treated HLA-DR showed identical distribution whether boiled or not, *i.e.*, no dimers were observed, only monomer bands and large amounts of degradation products smaller than 30 kDa. The boiled HLA-DQ isolate eluted at pH 11 showed monomer bands at 35 kDa (α -chain) and 31 and 29 kDa (β -chain), and the unboiled pH 11 isolate showed the dimer at about 60 kDa. The HLA-DQ isolate eluted at pH 2 showed fewer degradation products than the corresponding DR isolate, and the unboiled sample still showed a weak dimer band at 60 kDa. This observation points to a higher stability of the HLA-DQ dimer compared to that of the homologous DR dimer. Self-peptides stabilizing the dimer were bound to HLA-DQ, as ascertained with the RP-HPLC chromatogram of the 20-kDa ultrafiltrate of the pH 2-treated DQ isolate (data not shown). The dimer stability seems to vary not only between DR and DQ alleles but also between different DR alleles. *DR1* for example, in contrast to other DR alleles, is still capable of binding peptides in our binding/competition assay after being treated with pH 2.⁵

The HPLC Pattern of the HLA-DR-bound Peptides. The HPLC separation pattern of the HLA-DR-bound peptide pool is shown in

⁴ H. Kalbacher *et al.*, manuscript in preparation.

⁵ T. Halder, unpublished observation.

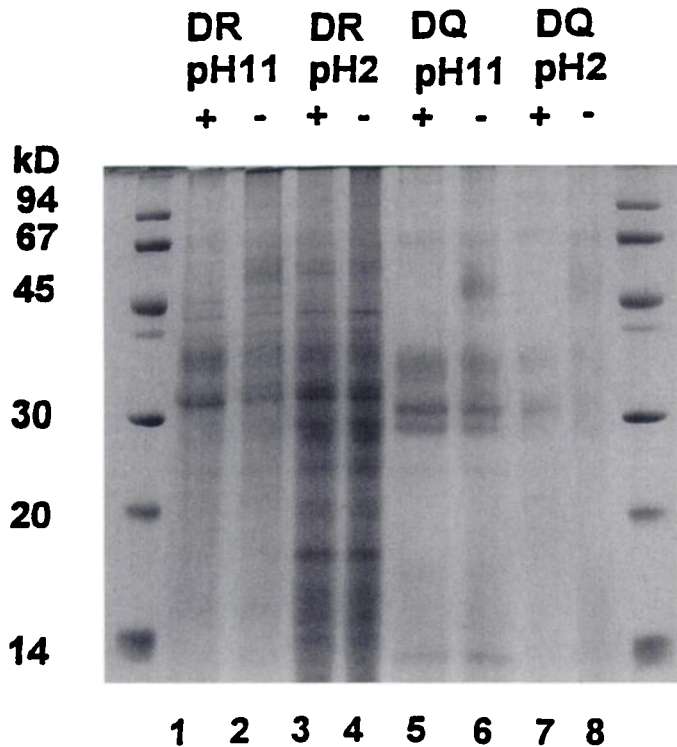


Fig. 1. A 13% SDS-PAGE analysis of HLA-D molecules isolated from the human melanoma cell line FM3. The HLA-DR pH 11 (Lanes 1 and 2) and pH 2 isolates (Lanes 3 and 4) as well as the DQ pH 11 (Lanes 5 and 6) and pH 2 isolates (Lanes 7 and 8) were observed after boiling for 5 min (+) or unboiled (-), respectively. The separation was performed under reducing conditions.

Fig. 2. The profiles demonstrate the complexity of the bound peptides, making it apparent that the FM3 HLA-DR molecules are able to bind at least hundreds of different peptides. Using detection at 295 nm, a variety of peaks containing tryptophan could be identified. Some fractions have been submitted directly to the sequencer, whereas others were first rechromatographed (*e.g.*, the peak at 42.8 min), because the separation by a single RP-HPLC run was not sufficient to get exactly one peptide/peak.

The First HLA-DR-bound Peptides from a Melanoma Cell Line. The melanoma cell line FM3, which expresses several known class I-restricted tumor antigens, was examined regarding its DR-associated peptides. From 1×10^{10} FM3 cells, we isolated about 1.0 mg (17 nmol) of HLA-DR and 350 μ g (6 nmol) of HLA-DQ molecules by immunoaffinity chromatography using the murine monoclonal antibodies L243 (α -DR) and T \ddot{U} 22 (α -DQ). The release of the DR-bound peptides resulted in about 10 nmol of peptides. After separation of the HLA-DR peptide pool, the most abundant sequenced peptides had 6–450 pmol. The sources of most of the sequenced peptides could be identified by aligning the sequences with the Swissprot protein sequence data base (Table 1). One of the peptides stems from the well-known invariant chain-derived CLIP region, position 80–103, and has already been found bound to DR molecules from other cell types, particularly human B-LCLs and processing mutants (T2 cells; Refs. 31, 34, and 35). It has been shown that CLIPs are remnants of the class II-associated invariant chain and that these peptides are the most dominant in antigen-processing mutants. Here we show that CLIP is also present in large amounts in the melanoma cell line FM3.

Another dominant peptide identified here was the 21-mer HLA-A2-derived peptide (position 62–82), which has also been observed by Chicz *et al.* (36) in isolates of the HLA-DRB1*0401/DRB4*0101 B-LCL Priess. The IFN- γ receptor peptide (position 128–144), now identified in

FM3, stems from a region of the protein that has also been found by Chicz *et al.* (36) in the B-LCL WT20, associated with HLA-DRB1*0301/HLA-DRB3*0201. Another peptide was derived from the transferrin receptor, position 616–632. A carboxyl-terminal truncated variant of this sequence (position 618–632) has been reported already, bound by HLA-DRB*0301 (37). A few other epitopes from the transferrin receptor are known from diverse B-LCLs and mouse cells, being restricted to the mouse class II allele *I-A^s*, HLA-DRB*0301, and HLA-DRB*1101 (38, 39). No DRB1*0201/DRB5*0101- or DRB1*0401-associated transferrin receptor epitopes had been published so far. In this context, it may be noted that we were also able to isolate another transferrin receptor-derived peptide from a patient with chronic myeloid leukemia (HLA-DRB*07/DRB1*1101).⁶ Because the transferrin receptor can be found in every dividing cell, it is unlikely that this peptide has a specific relation to the tumor.

More interestingly, we identified a sequence derived from hsp70 and a grp78 (BiP)-homologous protein in several HPLC fractions. According to the peptide mass and to our sequencing data, we found a grp78 homologous peptide 194–213, AVMRIINEPTAAAIAYGLDK, in which the asparagine in position 194 was replaced by alanine (*bold* letter). Peptides from this protein could be detected in two different HPLC fractions (see Table 1). Additionally, we identified a carboxyl-terminal truncated version of the original grp78 protein (194–210). We also found the corresponding peptide from the original hsp70 protein (hsp70, 168–?, NVLRIINEPTAAA...) but were unable to detect the carboxyl-terminus, because the sequence came from a rechromatographed peak that we did not submit to MALDI-MS. Hsp70 is known to be overexpressed in some forms of cancer, *e.g.*, microcytoma, lung carcinoma, and leukemia cells (40). Recently, it was reported that hsp70-reactive CD4⁺ T cells exist in several tumor tissues, including melanoma (41). These tumor-infiltrating lymphocytes recognize stressed cells and seem to play a Th1-like role that may support antitumor T-cell responses at local tumor sites. However, the hsp70 epitope recognized by these tumor-infiltrating lymphocytes has not yet been characterized. Three hsp70-derived peptides presented by HLA-DRB1*07 (DR7) and DRB1*1101/DRB3*0202 (DR11/DRw52) have been described thus far (30, 36). The present findings thus add additional variant self molecules to those accepted as tumor antigens or potential tumor antigens on melanoma cells, but in this case, with HLA-DR as the putative antigen-binding molecule.

In addition to peptides from these ubiquitous sources, we identified one DR-restricted peptide of the tumor antigen gp100 (Pmel17). This protein is well known to provide peptide sequences recognized by melanoma-specific CTLs that are presented by HLA-A2 on the melanoma cells. At least five antigenic peptides derived from gp100 that stimulate specific HLA-A2-restricted CTL clones have been previously identified: (a) YLEPGVTA (position 280–288); (b) LLDG-TATLRL (position 457–466); (c) KTWGQYWQV (position 154–162); (d) ITDQVPFSV (position 209–217); and (e) VLYRYGSFSV (position 476–485; Refs. 14 and 17–20). Thus far, however, no HLA class II-associated peptides of gp100 have been observed. We demonstrate here that the 16-mer gp100 peptide 44–59, WNRQLYPEW-TEAQRD, is a HLA-DRB1*0401-associated self-peptide of the melanoma cell line FM3. This sequence is derived from a completely different part of the molecule compared to the known class I-binding sequences. Because gp100 is highly expressed in melanoma cells, we suggest that the presentation of this epitope might be melanoma specific. The gene product of gp100 has been shown to be involved in melanosomal formation and is also recognized by mAb HMB45 routinely used in the detection of cells of melanocytic origin.

Another interesting sequence that we could identify as a potential

⁶ Unpublished observations.

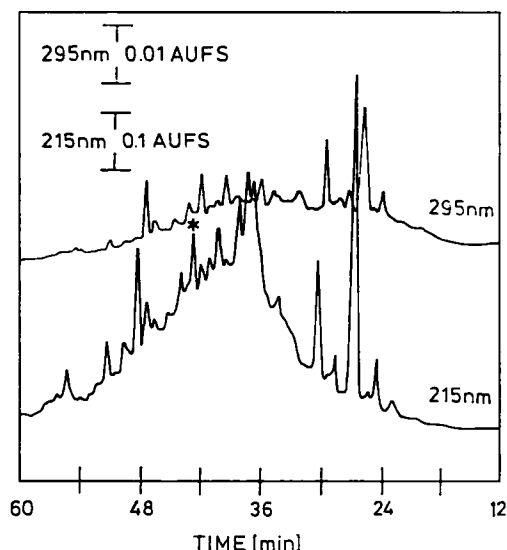


Fig. 2. RP-HPLC separation profile of the HLA-DR-associated peptide pool from FM3. The chromatogram represents the peptide repertoire as detected by UV absorbance for both 215 nm (peptide bonds) and 295 nm (content of the tryptophan residues) at full-scale absorbance of 100 mAU and 10 mAU, respectively. The plotting direction is from right to left. The peak labeled with an asterisk was submitted to rechromatography using a microcapillary column as described in "Materials and Methods."

tumor antigen presented by HLA-DR on FM3 is derived from annexin II (lipocortin II), position 208–223, DVPKWSIMTERSVPH. Annexin II is a protein that is present in most cells, but it is overexpressed in many types of tumor cells, *e.g.*, in glioblastoma multiforme (42) and pancreatic carcinoma (43). The function of annexin II has not yet been determined exactly, but it is known to be a Ca^{2+} -dependent phospholipid-binding protein that acts as a substrate for the src tyrosine kinase and possesses two different Ca^{2+} -binding sites (44). There is increasing evidence that annexin II plays a role in DNA synthesis and cell proliferation. Its expression is regulated during the cell cycle (45). For this reason, annexin II may be a potential candidate for a tumor antigen. Furthermore, there is evidence that CTAA 28A32-32K, an antigen related to the annexin family that is recognized by the human IgM 28A32 mAb, may be an important common colon tumor-associated rejection antigen (46).

Specific Binding of the Isolated Self-Peptides to the Solubilized HLA-DR Alleles of FM3. Specific binding of the gp100 epitope to isolated HLA-DRB1*0401 and to HLA-DRB1*0201/DRB5*0101 (DR2b/a) using an *in vitro* binding/competition assay was examined (Fig. 3a). The affinity of the peptide for DRB1*0401 ($\text{IC}_{50} = 7 \mu\text{M}$) was more than 10 times higher compared to DRB1*0201/DRB5*0101 ($\text{IC}_{50} = 90 \mu\text{M}$). This observation is in agreement with the known

peptide binding motif for HLA-DRB1*0401 (47). According to this, an aromatic or large hydrophobic residue is required as first anchor residue or position *i* (here, L); in position *i* + 3, M, A, V, and L are found preferentially (here, E). In position *i* + 5, a T or S is strongly favorable (here, T), and in position *i* + 8, small residues like A, S, G, or T are preferred (here, Q). Thus, the residues in relative positions *i* and *i* + 5 are optimal, the Q in position *i* + 8 is tolerated, and only the E in *i* + 3 is suboptimal. For the DR2a/b alleles present in the EBV-transformed B-cell line LD2B, there are two different peptide binding motifs (48). HLA-DRB5*0101 (DR2a) requires an aromatic or large hydrophobic residue in relative position *i*; Q, V, I, or M in position *i* + 3; and a charged residue in *i* + 7 or *i* + 8. HLA-DRB1*0201 (DR2b) prefers a hydrophobic residue in position *i* (L, V, or I), an aromatic or large hydrophobic residue in position *i* + 3, and an aromatic or hydrophobic amino acid in position *i* + 6. None of these motif requirements can be sufficiently met by the gp100 peptide. There are two possibilities for the first anchor *i* (L or Y), then at *i* + 3, we would have E or W; for *i* + 6, E or A; and for positions *i* + 7 or *i* + 8, A, Q, or R. Therefore, the only conceivable possibility for gp100 44-59 to bind to a DR2 allele, namely to DR2a, is using the Y as a primary anchor; then we have an R in position *i* + 8, but an unfavorable large residue (W) in position *i* + 3. The binding capacity of the identified annexin II peptide 208-223, in contrast, was extremely high for HLA-DRB1*0201/DRB5*0101 (IC_{50} of 0.19 μM) but was also very good for HLA-DRB1*0401 (IC_{50} of 2.3 μM ; Fig. 3b). Indeed, this peptide possesses the three different motifs for DR2a/b and DR4 perfectly. For DR2a and DR4, the anchor residues are W in position *i*, I in position *i* + 3, T in position *i* + 5, R in position *i* + 7, and S in position *i* + 8. For DR2b, residues V (position *i*), W (position *i* + 3), and I (position *i* + 6) are used. Therefore, the annexin peptide was likely to be bound to all three DR alleles, whereas the gp100 peptide was probably isolated from DR4. This assumption can explain why the annexin peptide was the most abundant peptide identified (370 pmol/ 10^{10} cells), whereas the gp100 peptide showed a yield of 17 pmol/ 10^{10} cells. In our competition assay, hsp70, 168-182, NVLRIINEPTAAAIA showed high affinity to HLA-DR4 with an IC_{50} of 3.5 μM and an intermediate affinity to HLA-DR2 ($\text{IC}_{50} = 50 \mu\text{M}$; Fig. 4, a and b). This can be explained with the postulated motifs for the different alleles (see above). For HLA-DR4, the isoleucine residue 172 is used as the primary anchor (*i*), in position *i* + 3, we have a glutamic acid, in the important position *i* + 5, we have an optimal threonine, and in position *i* + 8, we also have an optimal alanine residue (see **bold letters**). The glutamic acid in position *i* + 3 is suboptimal but is accepted. For DR2, there is no positively charged residue in this peptide that can satisfy the motif requirement perfectly. Therefore, it is reasonable that we observed only intermediate affinity for this allele. CLIPs are good

Table 1 Amino acid sequences of endogenous peptides eluted from HLA-DRB1*0401/*02x/DRB5*0101 of the human melanoma cell line FM3, determined by Edman microsequencing and MALDI-MS

Peak/Retention time (min)	Sequence	Source (human)	Residues	Mass (observed/[M + H] ⁺)	Yield/ 1×10^{10} cells (pmol)
37/38.5	VDDTQFVRFSDAASQRMEPR	HLA-A2 α -chain	62–82	2457.3/2470.7	450
43/42.8	AVMRIINEPTAAAIA YGLDK	grp78 (BiP) homologous	A194, 195–213	2115.3/2117.5	80
43/42.8 (rechro)	WNRQLYPEWTEAQRDL	gp100 (Pmel17)	44–59	2121.1/2105.3	17
43/42.8 (rechro)	NVLRIINEPTAAAIA...	hsp70	168–?	ND ^a	6
43/42.8 (rechro)	AVMRIINEPTAAAIA...	grp78 (BiP) homologous	A194, 195–?	ND	10
45/43.7	NVMRIINEPTAAAIA YG	grp78 (BiP)	194–210	1796.2/1804.2	45
45/43.7	VDDTQFVRFSDAASPREPRAPWIE	HLA-B7 α	62–87	3046.5/3034.3	50
48/46.0	LLSFVRDLNQRADIK	Transferrin receptor	616–631	1958.8/1951.3	30
48/46.0	QDQEFLKMLNLFYQT	Cytochrome P450 IIB6	192–206	1922.3/1918.2	12
48/46.0	YPHNFVXAGTAIQKY...	Unknown	?–?	ND	18
51/48.3	GPPKLDIRKEEKQIMID	IFN- γ receptor	128–144	2015.5/2010.4	10
51/48.3	DVPKWSIMTERSVPH	Annexin II	208–223	1905.3/1895.2	370
55/51.2	LPKPPKVPVKMRMATPLLMQALPM	Invariant chain	80–103	2683.4/2676.5	140

^a ND, not determined.

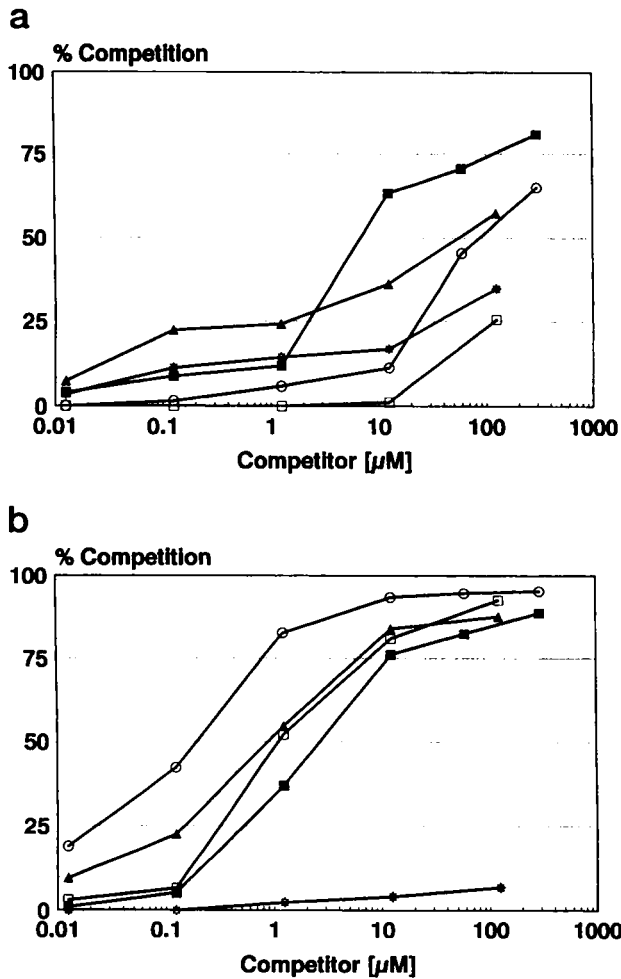


Fig. 3. Competition by gp100 44-59 (a) and annexin 208-223 (b) for binding to different HLA-DR alleles. The following fluorescent AMCA-labeled peptides were used at $1.5 \mu\text{M}$: HA, 306-318, PKYVKQNTLKLAT for DRB5*0101 (○), DRB1*0401 (■), and DRB1*0101 (▲); hsp65, 3-13, KTIAYDEEARR for DRB1*0301 (*); and hsc70, 239-250, NHFI-AEFKRKHK for DRB1*1101 (□). Binders, competitors, and HLA-DR isolates ($0.2 \mu\text{M}$) were incubated simultaneously with a mixture of protease inhibitors for 40 h at 37°C , and then separation was performed using a high-performance size-exclusion column.

DRB1*0201-binders ($\text{IC}_{50} = 1.0 \mu\text{M}$) but possess only low affinity to the DRB1*0401 allele, with an IC_{50} of $100 \mu\text{M}$ (Fig. 4, a and b). They usually use methionine 90 as primary anchor, ...KMRMAT-PLLMQ... (49, 50). For DR4, the proline in position $i + 5$ is not accepted, and the methionine in position $i + 8$ is too large. The affinity of the IFN- γ receptor peptide 128-144 to DR2 and DR4 proved to be very low ($\text{IC}_{50} > 1000 \mu\text{M}$; Fig. 4, a and b), whereas a carboxyl-terminal extended peptide (IFN- γ receptor 128-146) showed relatively high affinity for DR3, with an IC_{50} of $3.0 \mu\text{M}$ (37). The transferrin receptor sequence (position 618-632) has been shown to be a very potent DR3 binder possessing an IC_{50} of $0.7 \mu\text{M}$. In our competition assay, the transferrin receptor peptide 618-633, LLS-FVRDLNQYRADIK showed comparably high affinity for DR2 ($\text{IC}_{50} = 8.0 \mu\text{M}$) and DR4 ($\text{IC}_{50} = 3.3 \mu\text{M}$; Fig. 4, a and b). Both alleles should use the same peptide residues as anchors with valine 622 as the primary anchor. For DR4, the glutamine in position $i + 5$ is not optimal; for DR2a, the valine in position i is too small; and for DR2b, the leucine in position $i + 3$ is more unfavorable than an aromatic residue.

Peptide Binding to Other HLA-DR Alleles. Additional competition assays with other HLA-DR isolates showed excellent binding of

annexin II 208-223 to HLA-DRB1*0101 (DR1, $\text{IC}_{50} = 0.9 \mu\text{M}$) and HLA-DRB1*1101 (DR11, $\text{IC}_{50} = 1.1 \mu\text{M}$) but very weak affinity to HLA-DRB1*0301 (DR3; Fig. 3b). This is reasonable, because DR3 needs an aspartic acid in position $i + 3$ as an allele-specific anchor and does not accept a glutamic acid in this position (37). DR1 has a binding motif similar to that of DR4, also requiring an aromatic or large hydrophobic residue as primary anchor; M, L, I, A, Q, and E in position $i + 3$; a small residue in $i + 5$; and a hydrophobic but not aromatic or small residue in position $i + 8$ (47, 51, 52). Thus, the annexin II peptide uses the same residues for binding to DR1 and DR4. Binding of annexin 208-223 to DR11 is explicable by comparing its sequence (DVPKWISIMTERSVP) with the known DR11 binder influenza HA 306-318 (PKYVKQNTLKLAT). The anchor residues (*bold letters*) are very similar. The corresponding motif proposed by Newcomb and Cresswell (30) demands an aromatic residue as primary anchor and a positively charged residue in position $i + 7$. Another positively charged amino acid often occurs in position $i + 5$ but does not seem to be essential.

The gp100 peptide 44-59 (WNRQLYPEWTEAQRD) is also able to bind to DR1 ($\text{IC}_{50} = 55 \mu\text{M}$) but has only low affinity to DR3 ($\text{IC}_{50} > 500 \mu\text{M}$) and DR11 ($\text{IC}_{50} > 500 \mu\text{M}$), because there is no aspartic acid available in position $i + 3$ and no positively charged residue in position $i + 7$ (Fig. 3a).

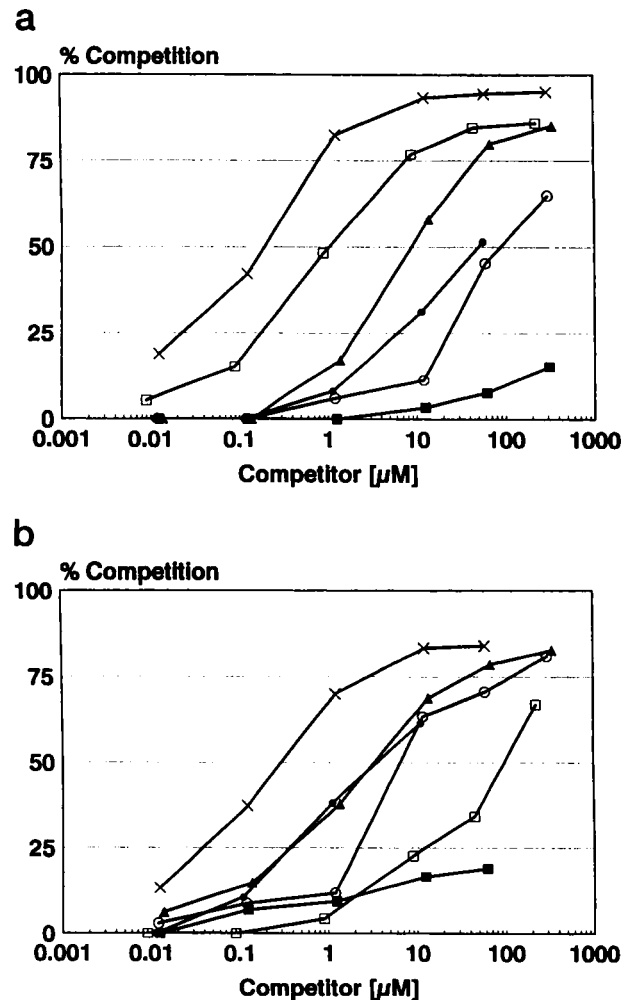


Fig. 4. Competition by different HLA-DR-bound FM3 self-peptides for binding to HLA-DR2 (HLA-DRB1*1501/DRB5*0101) using the fluorescent AMCA-labeled specific DRB5*0101 binder HA 306-318 (a) and binding to HLA-DR4 (HLA-DRB1*0401) also using AMCA-HA 306-318 (b). The highest concentration of hsp70 could not be determined due to solubility problems. ○, gp100 44-59; ■, g-IFN- γ 128-144; ▲, TFR 618-632; □, CLIP 80-104; X, annexin II 208-223; ●, hsp70 168-182.

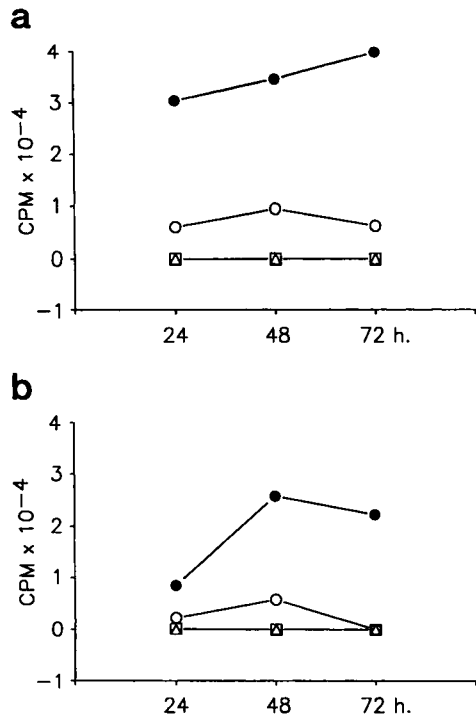


Fig. 5. T-cell sensitization to annexin II 208-223 (a) and gp100 44-59 (b). PBMCs from an HLA-DRB1*0401 and a normal donor were stimulated for 7 days and restimulated for another 7 days by CHO transfectants expressing HLA-DRB1*0401 and CD80 that had been pulsed with the annexin or gp100 peptide or not pulsed at all. Responding cells were restimulated with the same CHO cells pulsed either with annexin 208-223 or with the DRB1*0401-binding peptide gp100 44-59. The data show median cpm of triplicate cultures (incorporation of tritiated thymidine) after subtraction of the background values recorded using fixed CHO cells alone as stimulators. a, T-cell sensitization to annexin II 208-223 peptide. Tertiary responses of T cells sensitized against HLA-DRB1*0401/CD80-cotransfected CHO cells alone (negative controls □ and Δ) or pulsed with annexin peptide (● and ○) are shown. Annexin-sensitized cells were restimulated with annexin (●) or gp100 (○)-pulsed CHO cells. Negative controls were also restimulated with annexin (□) or gp100 (Δ)-pulsed CHO cells. Data represent mean thymidine incorporation of triplicate cultures (SE < 10%). b, T-cell sensitization to gp100 44-59 peptide. Tertiary responses of T cells sensitized against HLA-DRB1*0401/CD80-cotransfected CHO cells alone (negative controls, □ and Δ) or pulsed with gp100 peptide (● and ○) are shown. Peptide-sensitized cells were restimulated with gp100 (●) or annexin (○)-pulsed CHO cells. Negative controls were also restimulated with annexin (□) or gp100 (Δ)-pulsed CHO cells. Data represent mean thymidine incorporation of triplicate cultures (SE < 10%).

Annexin II 208-223 and gp100 44-59 Represent Epitopes Immunogenic for T Cells. To demonstrate T-cell sensitization to annexin II 208-223 and gp100 *in vitro*, we used an artificial APC that has previously been shown to provide excellent presentation of superantigen, antigen, and alloantigen in primary and secondary T-cell sensitization experiments (33). CHO cells expressing high levels of HLA-DRB1*0401 on their surfaces, together with the critical costimulatory ligand CD80, were pulsed with the annexin or the gp100 peptide, fixed, and cocultured with PBMCs from an HLA-DRB1*0401⁺ donor. As a negative control, unpulsed CHO cells were used in parallel experiments with the same donor. After 7 days of culture, followed by restimulation and an additional 7 days of culture, responding cells were harvested and restimulated with CHO APCs pulsed with the specific annexin II/gp100 peptide or with a control peptide known to bind well to the HLA-DRB1*0401 molecule (gp100/annexin II). As shown in Fig. 5a, annexin-pulsed CHO cells were able to sensitize responding T cells to the peptide. A very similar result was obtained for gp100-pulsed CHO cells (Fig. 5b). The response both to annexin 208-223 and gp100 44-59 was specific, because annexin-primed cells were unable to respond to CHO cells pulsed with gp100 peptide and *vice versa*. It was also dependent on the presence of the corresponding peptide in the sensitization cultures, because PBMCs

cocultured with unpulsed CHO cells were restimulated only at a very low level by annexin- or gp100-pulsed APCs.

DISCUSSION

The present results identify for the first time MHC class II HLA-DR-bound endogenous peptides expressed by melanoma cells. Because some of these peptide-MHC complexes have been shown to be recognizable by T cells, they may prove useful in the immunotherapy of melanoma. These results document that the same gp100 molecule giving rise to a known class I-restricted tumor antigen recognized by CD8⁺ T cells provides a potential class II-restricted tumor antigen, derived from a different portion of the same molecule, which is recognized by CD4⁺ T cells. The gp100 44-59 peptide defined here, however, displayed relatively weak binding to most HLA-DR alleles tested and therefore may not represent a widely applicable candidate for tumor vaccination. On the other hand, this peptide might contribute to tissue-specific alloreactivity (53). Another peptide identified in the present work, the annexin II 208-223 peptide, showed strong binding to all alleles except HLA-DRB1*0301 and may therefore be more generally applicable. Recently Wölfel *et al.* (54) described a mutated CDK4 gene product recognized by class I-restricted CD8⁺ melanoma-specific T cells. The point-mutated grp78 (BiP) homologue isolated from FM3 (Table 1) might therefore also be unique to the individual FM3 melanoma and might be recognizable by melanoma-specific T cells.

The cellular results demonstrate that the endogenous annexin II 208-223 peptide as well as the gp100 44-59 peptide eluted from the HLA-DR molecules of a melanoma cell line represent sequences that form epitopes recognizable by T cells (Fig. 5, a and b). We have thus far shown that the sensitized T cells are capable of autocrine proliferation. By measuring the cytokine levels of the supernatants of the sensitized T cells, we could clearly demonstrate that they belonged to the Th1 type secreting high amounts of IFN- γ and nearly no interleukin 4 (data not shown). This suggests that they may also be cytotoxic, and their class II restriction further implies that they are CD4⁺ cells. It remains to be confirmed that the annexin II and the gp100 epitopes are indeed tumor specific and that all or a proportion of native melanoma cells can present them on their surfaces. For the gp100 protein, we could demonstrate that epitope 44-59 seemed to play a similar role for CD4⁺ T cells as did other epitopes from the same molecule for CD8⁺ T cell recognition. Such MHC class II-restricted T cells may represent powerful antitumor effectors for use in adoptive immunotherapy of melanoma, either by themselves or perhaps preferably together with CD8⁺ cytotoxic effectors specific for the well-established class I-restricted melanoma antigens.

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