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Expression of the Mouse MHC Class Ib H2-T11 Gene Product, a Paralog of H2-T23 (Qa-1) with Shared Peptide-Binding Specificity

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The mouse MHC class Ib gene H2-T11 is 95% identical at the DNA level to H2-T23, which encodes Qa-1, one of the most studied MHC class Ib molecules. H2-T11 mRNA was observed to be expressed widely in tissues of C57BL/6 mice, with the highest levels in thymus. To circumvent the availability of a specific mAb, cells were transduced with cDNA encoding T11 with a substituted α3 domain. Hybrid T11D3 protein was expressed at high levels similar to control T23D3 molecules on the surface of both TAP⁺ and TAP⁻ cells. Soluble T11D3 was generated by folding in vitro with Qa-1 determinant modifier, the dominant peptide presented by Qa-1. The circular dichroism spectrum of this protein was similar to that of other MHC class I molecules, and it was observed to bind labeled Qa-1 determinant modifier peptide with rapid kinetics. By contrast to the Qa-1 control, T11 tetramers did not react with cells expressing CD94/NKG2A, supporting the conclusion that T11 cannot replace Qa-1 as a ligand for NK cell inhibitory receptors. T11 also failed to substitute for Qa-1 in the presentation of insulin to a Qa-1–restricted T cell hybridoma. Despite divergent function, T11 was observed to share peptide-loading specificity with Qa-1. Direct analysis by tandem mass spectrometry of peptides eluted from T11D3 and T23D3 isolated from HeLa cells demonstrated a diversity of peptides with a clear motif that was shared between the two molecules. Thus, T11 is a paralog of T23 encoding an MHC class Ib molecule that shares peptide-binding specificity with Qa-1 but differs in function. The Journal of Immunology, 2014, 193: 1427–1439.

M ajor histocompatibility complex class Ia molecules, including HLA-A, -B, and -C in human and H2-K, -D, and -L in mice, are expressed on the surface of most nucleated cell and present mainly endogenously derived antigenic peptides to CD8⁺ T cells, initiating signals required for the positive selection in the thymus and activation in the periphery. The assembly and cell-surface expression of MHC class I H chain with peptide and β2-microglobulin (β2m) is dependent on TAP and other components of the endoplasmic reticulum (ER)–localized peptide-loading complex (1).

Another group of MHC class I molecules, known as nonclassical or MHC class Ib, are encoded by genes mostly located at the telomeric end of the MHC gene region (2). Class Ib molecules have a similar structure, at both genomic and protein levels, to class Ia molecules, but class Ib molecules generally have more limited tissue distribution, lower expression levels, and fewer alleles in comparison with MHC class Ia molecules (2–4). The mouse MHC class Ib genomic region is further divided to three subregions, H2-Q, -T, and -M (2, 5). There are ~40 MHC class Ib genes present in the C57BL/6 (B6) mouse genome, and only about half of them were reported to be transcribed (3). A number of MHC class Ib molecules have been studied, and some have been shown to have specialized function, but the majority remain to be characterized. For example, H2-M3 preferentially binds N-formylated peptides that originate from bacterial or mitochondrial proteins, and it is not detectable on the cell surface until N-formylated peptide is bound (6, 7). H2-M3 has been reported to actively participate in anti–Listeria monocytogenes immune responses (8–12). By contrast, TL (encoded by T18) assemblies without bound peptide (13), and it serves as a ligand for CD8α, regulating the function of a subset of CD8α⁺ intestinal intraepithelial T cells (14, 15).

H2-T23 encodes one of the most well-studied MHC class Ib proteins, Qa-1 (16). The T23 gene is ubiquitously transcribed (3), but the surface expression level of Qa-1 is lower than that of the MHC class Ia molecules. There are a number of identified alleles, but most laboratory mouse strains express Qa-1b or Qa-1a, and other alleles are closely related to these prototypes (17–19). Unfortunately, the genes encoding Qa-1 are not mapped in strains other than C57BL/6 and BALB/c; therefore, we do not know if they are allelic. Some of these Qa-1 molecules might be encoded by paralogous genes derived from a strain-specific gene duplication of the T23-like ancestral gene. Qa-1 appears to have a highly selective peptide-binding specificity, predominantly loading with Qa-1 determinant modifier (Qdm; AMAPRTLLL), a peptide derived from the conserved leader sequence of H-2D and H-2L class Ia molecules (20, 21). Despite its origin in leader sequences, loading of Qdm is dependent on TAP, as well as tapasin and presumably other component of the class I peptide-loading complex (4, 22). The fragment of the leader sequence that contains Qdm is released into the cytoplasm after cleavage by signal...
peptidase and signal peptide peptidase, thus requiring TAP for transport into the ER lumen. Qa-1–Qdm complex functions as the sole ligand for CD94/NKG2 inhibitory and activating receptors on NK cells and recognition by CD94/NKG2 is highly specific for the sequence of bound Qdm peptide (23, 24). The expression of Qa-1–Qdm serves as a quality-control system, such that cells lacking components of the peptide loading machinery required for generation of Qa-1–Qdm are killed by CD94/NKG2A+ NK cells (25).

Although Qdm is the dominant peptide presented by Qa-1 molecules, it is evident that Qa-1 has a capacity to present other peptides to CD8+ T cells. Qa-1–specific T cells have been reported to participate in immune responses to L. monocytogenes (9, 26) and Salmonella typhimurium (27, 28), and Qa-1–restricted T cells with specificity for proinsulin (29) and insulin (30, 31) have been characterized. A number of studies have reported a role for Qa-1–restricted CD8+ T cells in regulating immune responses and self-tolerance (32–35) and in immune surveillance of TAP-deficient tumors (36, 37). Recently, Nagarajan et al. (38) have demonstrated a role for Qa-1–restricted CD8+ T cells in monitoring the function of ERAAP, an aminopeptidase that mediates trimming of peptides presented by MHC class I molecules in the ER. Cytotoxic effector cells were shown to recognize a self-peptide (FL9) that is selectively presented by Qa-1 in ERAAP-deficient cells.

The MHC is shaped by successive rounds of segmental duplications. The mouse H2-T region, where Qa-1 is encoded, contains ~20 class I genes. This number varies greatly among haplotypes due to strain-specific deletions/duplications. The H2-T region of C57BL/6 and BALB/c contains two and A/J mice contain three highly similar segments (39–42). These duplicated segments were further modified by monogenic duplications, deletions, and single nucleotide changes, leading to strain-specific class I gene pseudogene content. This process led to variable numbers of T23/T11-, T22/T18-, T25-, and T18/T3-like paralogous genes, pseudogenes, and gene fragments (43). For example, the TL Ag (43), which is expressed on intestinal epithelium and thymocytes, can be encoded by one (H2-T3), two (H2-T3 and -T18), or three genes (in A/J), due to strain-specific deletions/duplications. The H2-T region of the mouse genome and total RNA were as follows: T11 forward, 5'-AGTATTTTCACACCTGTCGTA-3'; T11 reverse, 5'-TAGGATATGCGAGCTTAATGTG-3'; T23 forward, 5'-AGTATTTTCACACCTGTCGTA-3'; and T23 reverse, 5'-AACGACCTCAGGGTGACTTCAT-3'. PCR was performed using Takara Taq polymerase from Clontech.

Murine RNA polymerase 2A (POLR2A) was used as the reference gene for quantitative PCR (qPCR) analysis of T11. The primers for qPCR were: T11 forward, 5'-TAACCTGAGGAGCCCTGTCC-3'; T11 reverse, 5'-TAGGCCCCCTGGACAACTACC-3'; POLR2A forward, 5'-GACAAACGTGGCTCCTGGC-3'; and POLR2A reverse, 5'-GGCTT-GGCCCTCTGATCTCG-3'. The mouse tissues were collected and stored in RNA later solution (Ambion) for less than a week at 4°C before RNA was extracted. The total RNA was extracted using the RNeasy mini kit (Qiagen). The cDNA was synthesized using QuantiTect Reverse Transcription kit (Qiagen). The qPCR kit Absolute QPCR SYBR Green Mix (Thermo Scientific) was used in the qPCR analysis, which was performed on a Lightcycler 480 system (Roche).

**Generation of hybrid H2-T11 and H2-T23 molecules**

The α3 domain of H2-T11 or H2-T23 cDNA was replaced with the α3 domain of the H2-Dβ cDNA, and the hybrid molecules were named as H2-T11D3 or H2-T23D3, respectively. The H2-T11D3 and H2-T23D3 cDNAs were synthesized at Biomatik. The synthesized cDNAs were verified by sequencing before being cloned into expression vectors. The cDNAs were cloned into a retroviral vector MigR1 for expression in mammalian cells (45). The soluble forms (lack of the transmembrane and cytoplasmic domains) of H2-T11D3 or H2-T23D3 were generated by PCR and cloned into a bacteria expression vector pET29pf (the NIBR Selector Core). The cloned products were verified by restriction enzyme digestion and sequencing. All restriction enzymes were from New England Biolabs. The plasmids were purified using the Plasmid Mini Kit (Qiagen) for digestion and cloning and the EndoFree Plasmid Maxi Kit (Qiagen) for transfection.

**Abs, flow cytometry, and cell sorting**

Purified anti–Qa-1b (6A8, mouse IgG1, κ), anti-Dβ3 domain (28-14-8s, mouse IgG2a, κ), and anti-human β2m (BB7.7) Abs were purified from hybridoma supernatants using protein A affinity chromatography. FITC-labeled anti-mouse CD3ε (145-2C11), PerCP-Cy5.5-labeled anti-mouse B220 (RA3-6B2), PE-labeled anti-mouse NKp46 (29A1.4), and PE-Cy7–labeled anti-mouse NK1.1 (PK136) Abs were purchased from BioLegend or BioScience, or Bio Probe. The TD-Aba was diluted in a buffer composed of PBS, 0.5% BSA, and 2 mL EM. The suspended cells were incubated with the Abs and the tetramer for 20 min at 4°C. The stained cells were washed twice with the above buffer and fixed with 1% paraformaldehyde. The fluorescence was detected on a FACS Canto II (BD Biosciences). The data were analyzed by FlowJo (Tree Star), allophosphocyanin-labeled TCRβ (H57-597), PE- or allophosphocyanin-labeled anti-mouse B220 (RA3-6B2), FITC- or PerCP-Cy5.5-labeled anti-mouse CD4 (GK1.5), FITC- or PE-labeled anti-mouse CD8α (53-6.7), FITC-labeled anti-mouse CD8β (28-14-8s, HL3), and PE-labeled anti-mouse NK1.1-PE (PK136) were purchased from BioLegend, BioScience, or BD Biosciences and used for sorting of CD4+ CD8− double-negative, CD4+CD8+ double-positive (DP), CD4+ single-positive (SP), CD8+ SP, B cells, and dendritic cell (DC) subpopulations from thymus as well as T cells, CD4+ T cells, CD8+ T cells, B cells, DC, NK, and NKT subpopulations from spleen. The cell sorting was performed using a BD FACS Aria III (BD Biosciences).

**Cell culture**

Phoenix-GP, Hela, and T2 cells were cultured in DMEM complete media supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml t-glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, and 55 μM 2-ME (all from Invitrogen). Splenocytes were cultured in RPMI 1640 complete media supplemented the same way as the DMEM complete media. To stimulate splenocytes, cells were cultured with plate-bound anti-CD3 (28-14-8s, 37.57.1) and anti-CD28 (37.57.1) Abs in RPMI 1640 complete media. All cells were maintained at 37°C in a humidified cell-culture incubator containing 5% CO₂.

**Retroviral transduction**

Phoenix-GP cells were added at 4 × 10⁵ cells/dish to 6-cm collagen I-coated cell-culture dishes (BD Biosciences). Eighteen hours later, 10 μg MiGR1 empty vector or MiGR1 containing the T11D3 or T23D3 cDNA was cotransfected with 5 μg Env plasmid into the Phoenix cells by calcium-phosphate precipitation as previously reported (46). Two days later, the supernatant containing the packaged retrovirus was harvested and filtered through 0.45-μm sterile filters (BD Biosciences) before transduction. Half of a milliliter of 1 × 10⁶/ml Hela or T2 cells was mixed with 0.5 ml retroviral supernatant in the presence of 5 μg/ml...
polybrene (Sigma-Aldrich), and the cells were dislodged evenly on collagen I-coated 6-cm dishes. Four hours later, 4 ml fresh DMEM complete media was added. After at least 3 d, the cells were harvested, and the enhanced GFP (EGFP) expression was examined by flow cytometry. The transduced cells were sorted at least twice according to the EGFP expression level for cells stably expressing high levels of target genes.

Expression of soluble recombinant proteins

The pTCF vector containing the T11D3 or T23D3 genes was transformed into BL21 (DE3) Escherichia coli (Invitrogen). T11D3- or T23D3-positive clones were verified for producing recombinant proteins before they were expanded in 2 l Luria broth media. Isopropyl β-D-thiogalactoside (1 mM) was added to the culture when the OD600 reached ~0.6. The bacteria were harvested 4 h later. The bacteria were pelleted by centrifugation, resuspended in resuspension buffer (50 mM [pH 8] Tris-HCl, 25% [v/v] sucrose, 1 mM EDTA, 0.1% [w/v] Na azide, and 10 mM DTT), and stored at −80°C. The frozen bacteria were thawed, and 1 mg/ml lysozyme, 5 mM MgCl2, 33 μg/ml Dnase I, 3.3% (v/v) Triton X-100, and 10 mM DTT were added. The bacteria were stirred and lysed at room temperature for 1 h before sonication. The inclusion body was washed multiple times with wash buffer (50 mM [pH 8] Tris-HCl, 0.5% Triton X-100, 100 mM NaCl, 1 mM EDTA, 0.1% Na azide, and 1 mM DTT) until the protein pellet was white and the supernatant was clear. The pellets were washed one more time with the same wash buffer without DTT, and the recombinant inclusion body was solubilized in 6 M guanidine chloride, analyzed using 12% SDS-PAGE, aliquoted, and stored at −80°C until use.

In vitro MHC class I folding

Soluble recombinant T11D3 and T23D3 proteins were folded in vitro as described before (25). Briefly, 18 mg inclusion body of T11D3 or T23D3 H chain together with 6 mg human β2m L chain was diluted in 100 mM Tris folding buffer (pH 8) containing 400 mM l-arginine, 2 mM EDTA, 0.5 mM oxidized glutathione, 5 mM reduced glutathione, and 0.2 mM PMSF in the absence or presence of Odm peptides. The gram ratio of H chain/L chain peptide was 3:1:1. The folding reaction was performed at 10°C for 2 d before it was harvested and concentrated by Amicon ultrafiltration cells (Millipore). The concentrated sample was filtered through a 0.45-μm filter and purified by an S300 gel filtration column. The purified folded products were concentrated by Amicon Ultra centrifugal filters (Millipore), buffer exchanged with PBS, and stored at −80°C. The MHC folding products were analyzed using 4–20% gradient Tris–HCl PAGE (Bio-Rad).

Ag presentation assay

Transduced Hela cells (5 × 10⁵) or B6 splenocytes (1 × 10⁶) were used as APCs and cocultured with 1 × 10⁵ 6C5 T cell hybridoma cells (31) overnight in the presence of different doses of bovine insulin (Sigma-Aldrich) in 96-well plates. The supernatant was harvested, and the production of IL-2 was measured by Eu-based immunoassay (47).

Eu-based peptide binding assay

The peptide binding capacity of folded recombinant T11D3 or T23D3 was examined using an Eu-based immunoassay (47). Briefly, a 96-well ELISA plate was coated with 1 μg/ml anti-human β2m Ab (clone BB7.7) at 37°C for 2 h; the plate was blocked by 200 μl MTB (5% powdered skim milk, 1% BSA, and 0.01% Na azide) in TTBS buffer [50 mM Tris, 150 mM NaCl, and 0.1% Tween-20 (pH 7.5)] for 30 min at room temperature; 1 μg/mL folded MHC monomers was diluted in 100 μl MTB and incubated in the Ab-coated plate for >2 h at 4°C, the biotin-labeled peptides were diluted in PBS with 0.01% Nonidet P-40 (NP-40) and incubated the plate overnight at room temperature. The plate was washed extensively using Tween 20 and Tris-bufffered saline before the addition of the next reagent. Finally, the plate was developed using Europium reagent, and the fluorescence signal was recorded by a Victor V plate reader (PerkinElmer).

Fluorescence polarization assay

The folded MHC monomers were incubated with Alexa Fluor 488–labeled Qdm-4C (ANACRTLLL) peptides in citrate/phosphate buffer (200 mM citric acid and 250 mM Na2HPO4 [pH 7.5]). The parallel and perpendicular fluorescence signals (IP and LP) were recorded at 60-μs intervals for a total of 60,000 s by an Infinite F200 microplate reader (Tecan) at 37 or 25°C. Pure MHC monomer, Alexa Fluor 488–labeled peptide, or buffer alone was also detected independently to record the background signals. After the background signals were subtracted, the anisotropy was calculated according to the following formula: A = (IP−LP)/(IP+LP) (48).

Circular dichroism assay

The circular dichroism (CD) spectrum was measured by an Aviv 410 CD instrument (AVIV Biomedical). The folded MHC monomer concentration was determined by OD280 and diluted to 250 μg/ml in PBS. The far-UV CD spectrum was recorded in a cuvette with 1-mm path length at 25°C. The sample was scanned from 200–260 nm with a step of 1 nm. The averaging time was 3 s, and every step was scanned three times. The PBS background data were recorded in the same cuvette and subtracted from the sample data. The thermal stability of the folded MHC was measured by detecting the CD signal at 222 nm. The temperature was increased by a step of 2°C from 25 to 79°C. At each temperature point, the sample was equilibrated for 30 s before collecting the data. The averaging time was 30 s.

MHC class I tetramer preparation

T11D3 and T23D3 MHC tetramers were prepared as described before (49). The S300 purified MHC monomer was buffer exchanged to 10 mM Tris buffer (pH 8) and concentrated to 2 mg/ml. One milligram MHC monomer (8/10 volumes) was mixed with 1/10 volume of 10× BiomixA, 1/10 volume of 10× BiomixB, and 5 μg BirA enzyme (GeneCopoeia). The reaction was kept at 25°C overnight. The product was further purified by affinity chromatography using hybridization exchange (GEX) resin, and the recombinant inclusion body was snap-frozen and stored at −80°C. When tetramer was generated, the biotinylated MHC monomer was thawed and tetramerized by gradually adding APC-labeled streptavidin to it. The final molar ratio of biotinylated MHC to streptavidin was 4:1. One tenth of the total required allolphycocyanin-labeled streptavidin was added to the sample each time, and the sample was incubated in dark at room temperature for 10 min after each addition, so that each streptavidin was saturated. The tetramers were stored at 4°C and used within 6 mo.

Peptide elution and identification

The T11D3 and T23D3 bound peptides were eluted and identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described (50). Briefly, Hela-MigR1, Hela-T11D3, and Hela-T23D3 cells were cultured in 20–25 T150 cell-culture flasks, and the cells were harvested at ~80–90% confluence. The total cell numbers collected for each cell type were >1 × 10⁸. The cells were pelleted, washed twice with cold Dulbecco’s PBS, and stored at −80°C until lysing within 2 mo. The cells were lysed in NP-40 buffer (0.5% NP-40, 500 mM Tris-HCl [pH 8], 150 mM NaCl, and protease inhibitors). The lysate was centrifuged, and cell debris was removed. The supernatant was passed through a Tris-blocked Sepharose column to preclar the lysate. The hybrid MHC was immunoprecipitated by 24-8-ss cross-linked on Protein A beads. The beads were washed with four different buffers sequentially: 1) 0.005% NP-40, 50 mM Tris-HCl (pH 8), 150 mM NaCl, and 5 mM EDTA; 2) 50 mM Tris-HCl (pH 8) and 150 mM NaCl; 3) 50 mM Tris-HCl (pH 8) and 450 mM NaCl; and 4) 50 mM Tris-HCl (pH 8). The MHC–peptide complexes were eluted from the beads by 10% acetic acid, lyophilized, resuspended in 100 μl DMSO, loaded, and fractionated by HPLC (Beckman Coulter) into 27 fractions. Each fraction was concentrated to ~10 μl before mass spectrometry. The sequences of the eluted peptides were identified by LC-MS/MS.

Mass spectrometry analysis of peptide eluted from T11D3 or T23D3 was performed as previously reported. Briefly, 1 to 2 μl each HPLC fraction was analyzed by MS/MS (Agilent 6510 quadrupole-time-of-flight instrument with Chip Cube electrospray ionization; Agilent Technologies). The samples were injected using nanomass protein chip number 1 (40-μl trap, 75 × 43 mm, C18-BSR-ZX chip, 5-nm particles) at a flow rate of 400 nl/min. Data acquisition was done using MassHunter (version B.01.03) in a 2-GHz extended dynamic range at a rate of three scans per second followed by data-dependent MS/MS fragment scans of the three most intense ions. Precursor ion exclusion was set for 12 s after two consecutive MS/MS scans. Before each experiment, the quadrupole time-of-flight analyzer was tuned to a resolution of >12,000, and mass accuracy was calibrated to <2 ppm. Acquired MS/MS spectra were searched with no enzyme specificity using Spectrum Mill (Agilent Technologies) against the UniProt human FASTA protein database (August 2007 download). Raw peptide data files generated were converted into Excel format (Microsoft) and sorted according to their corresponding mass-to-charge ratio values, charge state, retention time, and intensity (50, 51). A user-defined intensity threshold (7.0) above the background noise was fixed to limit false-positive identification. All identified peptides sequences above this score were manually verified (51). In addition, peptides found in the fractions from control Hela-MigR1 lysates were considered contaminants and subtracted from the final list of peptides.
Results

Transcription of T11 gene in B6 and T23/Qa-1b knockout mice

H2-T11 and H2-T23 genes appear to have been duplicated from a common ancestor gene in evolution (42). Because the genomic sequence of the T11 from B6 mice is not available from the National Center for Biotechnology Information database, we cloned and sequenced the T11 genomic region from B6 mice using the H2-T11 locus genomic DNA sequence from 129 mice as the template (A. Kumánovics, unpublished observations). The H2-T11 and H2-T23 cDNA sequences showed a high degree of homology with sequences of Qa-1b, Qa-1a, and T11 ectodomains. The amino acid sequences of Qa-1b, Qa-1a, and T11 ectodomains were aligned, using CLUSTALW, SDSC Biology Workbench 3.2 (http://workbench.sdsc.edu).

The amino acid sequences of Qa-1b, Qa-1a, and T11 ectodomains were fully conserved; *, strongly conserved; :, weakly conserved.

Furthermore, CD4^-CD8^- double-negative, CD4^+CD8^+ DP, CD4^+ SP, CD8^+ SP, B cells, and DC subpopulations as well as T cells, CD4^+ T cells, CD8^+ T cells, B cells, DC, NK, and NKT subpopulations were sorted from thymus or spleen of Qa-1^-/- mice, respectively. Expression levels of H2-T11 of those subpopulations were compared by qPCR and shown in Fig. 2D (thymus, top panel, and spleen, bottom panel). Expression levels are similar across all tested cell subpopulations with the exception of DP thymocytes, which express relatively high levels of T11 mRNA. Upon activation by TCR engagement with CD3 and CD28 Ab, surface Qa-1 expression on splenocytes of B6 mice was increased, as reported previously. Similarly, using qPCR, T23 transcripts were also increased after activation. T11 transcripts from Qa-1^-/- splenocytes were increased by activation as well, but with delayed kinetics, peaking on days 3 and 4 (Fig. 2E).

Expression of H2-T11 encoded protein on the surface of TAP^+/+ and TAP^-/- cells

Our PCR and sequencing results indicated that T11 might encode a functional protein. We generated cDNA expression constructs encoding a chimeric T11 protein substituted with the α3 domain of H2-D^b to circumvent the absence of an mAb that recognizes T11 (37). The H2-D^b α3 domain is recognized by mAb 28-14-8s. Chimeric Qa-1 molecules were generated as a control, and these hybrid molecules were designated H2-T11D3 and H2-T23D3, respectively. The hybrid T11D3 and T23D3 were transduced into Hela (TAP^+) and T2 (TAP-deficient) cells using the MigR1 retroviral transduction system, which has an EGF reporter gene following an internal ribosome entry site (45).

High levels of T23D3 were detected on the surface of Hela cells with mAb 28-14-8s demonstrating that the chimeric Qa-H1 chain efficiently assembles with endogenous human β2m to form a stable complex (Fig. 3A, Hela-T23D3 panel). As previously reported for wild-type Qa-1 (23, 25, 53), cell-surface T23D3 can also be expressed at high levels in TAP-deficient cells (Fig. 3A, T2-T23D3 panels). Presentation of the dominant Qa-1–associated peptide Qdm is strictly TAP dependent (20, 53). However, Qa-1 has been reported to assemble with alternative non-Qdm peptides in TAP-deficient cells (37, 55). Cell-surface T23D3 was also detected with mAb 6A8, which recognizes an epitope in the α3 domain of Qa-1^b (Fig. 3A, T2-T23D3 panels).

FIGURE 1. Alignment of amino acid sequences of Qa-1^b, Qa-1^a, and T11 ectodomains. The amino acid sequences of Qa-1^b, Qa-1^a, and T11 ectodomains were aligned, using CLUSTALW, SDSC Biology Workbench 3.2 (http://workbench.sdsc.edu). α1, 1, 2, and 3 domains are boxed. *, fully conserved; :, strongly conserved; :, weakly conserved.
The chimeric T11D3 protein was expressed at levels similar to T23D3 on the surface of both Hela and T2 cells, as determined by mAb 28-14-8s staining (Fig. 3A, top panel). Significantly reduced staining was observed with the anti-Qa-1 b mAb 6A8, suggesting that the epitope recognized by this mAb is not fully preserved in T11. These results suggest that T11 can efficiently assemble and be expressed as a stable cell-surface protein, with the caveat that the α3 domain has been substituted in our experiments. Like T23D3, high levels of T11D3 were expressed in TAP-deficient T2 cells. It is possible that T11, like TL (T18d), assembles without bound peptide Ag (13, 56). Alternatively, like Qa-1, T11 may assemble with peptides through a TAP-independent mechanism.

**T11 cannot substitute for Qa-1 in T cell Ag presentation**

Previous findings from our laboratory showed that a subset of CD8 T cells with specificity for insulin is selected by Qa-1 b in mice (30, 31). The Qa-1 b–restricted CD8 T cell hybridoma 6C5 recognizes an epitope in the B chain of insulin. Hela cells expressing the chimeric Qa-1 T23D3 molecules were able to efficiently present insulin to 6C5 T cells, with even greater function than B6 splenocytes (Fig. 3B). Thus, the substituted α3 domain does not affect the Ag presentation function of Qa-1 from these T cells. By contrast, no T cell response was observed in experiments with Hela cells expressing similar levels of T11D3. We concluded that T11 cannot substitute for Qa-1 in Ag presentation to insulin-specific 6C5 T cells.

**Folding of soluble T11D3 with or without Qdm peptide in vitro**

The class Ia leader sequence-derived peptide Qdm has an optimal sequence for binding to Qa-1 b (24, 57), and it is the dominant peptide bound to Qa-1 molecules in TAP-expressing cells (21). As previously demonstrated for wild-type Qa-1 b (23, 24), T23D3 H chain assembles efficiently under standard folding conditions in vitro in the presence of Qdm peptide and β2m based on size-exclusion chromatography (Fig. 4A). The total RNA was extracted from unstimulated or stimulated B6 or Qa-1 b−/− splenocytes. The qPCR for T23 used RNA from B6 or for T11 used RNA from Qa-1 b−/− splenocytes, respectively. The qPCR was done as above, in triplicates (n = 3), and the experiment was repeated twice with similar results. One surface staining of Qa-1 is shown. DN, double-negative.
Qa-1, we set up folding reactions with T11D3 under identical conditions. A large peak corresponding to folded MHC class I molecules was observed with T11D3 in the presence of Qdm. In contrast to T23D3, a peak corresponding to folded T11D3 protein was also observed in the absence of Qdm, although the yield was lower than that observed in the presence of Qdm. The appropriate peaks from size-exclusion chromatography were concentrated and examined by SDS-PAGE. Both H and L chains of MHC class I were detected, indicating the assembly of T11D3 with β2m, even in the absence of Qdm (Fig. 4B). The products were also analyzed using an Eu-based fluorescence immunoassay (Fig. 4C). In this assay, the folding product was captured with an anti-β2m mAb (BB7.7), washed, and detected by 28-14-8s (anti-Dbα3) or 6A8 (anti-Qa-1β) mAbs. The results showed that folded T11D3 and T23D3 products can be recognized by both 28-14-8s and 6A8 Abs, further confirming assembly with β2m.

To further characterize the structure of the folded T11D3 and T23D3 proteins, CD was used to analyze secondary structure. The CD results showed that all three folded proteins—T11D3-β2m, T11D3-β2m-Qdm, and T23D3-β2m-Qdm—displayed wavelength spectrums (Fig. 4D) similar to those previously published for MHC class I molecules (13, 58–60). The CD spectrums each showed a single maximum signal at ∼220 nm, which was in conformity with a β-sheet–dominated structure in the MHC H chain. Thermal stability studies showed that folded T11D3 products had abnormal stability profiles (Fig. 4E). For most MHC class I proteins, the molecule loses its regular secondary structure, forming random coils, as the temperature increases, such that the molar ellipticity gradually approaches zero (58, 59, 61). With increasing temperature, T11D3 displayed evidence of denaturation, as shown by an increased molar ellipticity, but the denaturation was not complete at 80˚C. Instead, the molar ellipticity stabilized at ∼50˚C, possibly reflecting entry into a relatively stable misfolded conformation.

The capacity of chimeric T11 and T23 proteins to bind Qdm peptide

The peptide binding capacity of folded T23D3 and T11D3 was evaluated using biotin-labeled Qdm in Eu-streptavidin–based immunoassays. Folded proteins were incubated for 18 h at room temperature in microtiter wells coated with anti-β2m capture mAb by guest on April 21, 2017 http://www.jimmunol.org/ Downloaded from

FIGURE 3. Expression of hybrid T11D3 and T23D3 molecules and test of the function of hybrid molecule–expressing cells as APCs. (A) FACS analysis of hybrid T11D3 and T23D3 on the surface of Hela and T2 cells. Transduced Hela cells were stained with 28-14-8s (α-Dbα3), and T2 cells were stained with 28-14-8s and 6A8 (α-Qa-1β), respectively, shown as marked (red lines). The staining of isotype control Ab is blue. (B) Ag presentation assay to test capability of the hybrid MHC class Ib molecule–expressing cells to present insulin to 6C5 T hybridoma cells specific to bovine insulin (blns). The assay was set up in triplicates (n = 3), and the experiment was repeated twice with similar results. One of them is shown. spl, spleen.
replaced by the labeled peptide. Previous results have indicated that Qdm dissociates from Qa-1 with a $t_{1/2}$ of 40–100 min at 37˚C (25), a relatively rapid rate of dissociation despite having an optimal sequence for binding to Qa-1 (24). The observed peptide association rates probably reflect rate of dissociation of Qdm, a step necessary for binding of labeled peptide.

The kinetics of binding of labeled Qdm to T11D3-Qdm were highly unusual, displaying a rapid initial kinetics followed by a rapid decay in binding signal. This pattern suggests that T11D3-Qdm complexes are unstable at 37˚C, rapidly undergoing denaturation or conversion to a peptide unreceptive conformation. Consistent with the results of Eu–streptavidin peptide binding experiments, T11D3 that was folded in the absence of peptide showed little or no peptide binding activity. This protein may initially bind some labeled Qdm, but it very rapidly assumes a peptide unreceptive state. Complexes formed with labeled peptide and T11D3-Qdm were considerably more stable at 25˚C relative to 37˚C, yet the unusual decay in signal was still observed at this temperature (Fig. 5B, right panel). Overall, the results indicated that T11D3 can bind Qdm peptide, but T11D3-Qdm complexes are highly unstable and subject to denaturation or conversion to a peptide unreceptive conformation.

Evidence that T11 is not a ligand for CD94/NKG2 receptors

Qa-1 plays a key role in regulating NK cell activation as the exclusive ligand for CD94/NKG2 signaling receptors, which are expressed on a major fraction of NK cells, NKT cells, as well as a subpopulation of CD8+ T cells. CD94/NKG2 receptors display a high degree of specificity for the sequence of Qa-1–bound peptide, specifically recognizing Qdm (24). Several Qa-1 alleles have been identified, and they appear to share the capacity to bind Qdm and serve as ligands for CD94/NKG2 receptors (19). Given the similarity of T11 to Qa-1 and the capacity of T11D3 to bind Qdm peptide, we were interested in determining whether T11 could serve as an alternative ligand for CD94/NKG2.

CD94/NKG2 ligand binding can be demonstrated by flow cytometry using tetramers generated from in vitro–folded Qa-1–Qdm complexes to stain NK cells (23, 24). Similar to wild-type Qa-1, tetramers generated with chimeric T23D3-Qdm molecules were observed to stain a major fraction of CD3-NKp46+NK cells from the spleen and liver of B6 mice (Fig. 6). Thus, substitution of the T23 a3 domain does not disrupt receptor recognition. A substantial fraction of CD3+ lymphocytes from liver was also tetramer positive, reflecting the large number of CD94/NKG2+ NKT cells present in liver. By contrast, no staining was observed with tetramers generated from T11D3-Qdm molecules. The T11D3 molecules used to generate the tetramers were confirmed to contain β2m and Qdm, and they were appropriately biotinylated and tetramerized with streptavidin (data not shown). Nevertheless, it is possible that a fraction of T11D3 tetramers had loss of function from dissociation of Qdm. Despite this caveat, our results support...
the conclusion that T11 cannot substitute for Qa-1 as an alternative ligand for CD94/NKG2 receptors. In addition, no T11D3 tetramer-positive lymphocytes were identified in lymphocytes from thymus, inguinal lymph node, mesenteric lymph node, Peyer’s patches, or bone marrow (data not shown).

Analysis of peptides eluted from T11D3 and T23D3 expressed in Hela cells

T11D3 (like T23D3) can be expressed at high levels on the surface of TAP-deficient T2 cells, and some degree of folding of T11D3 H chain and β2m was observed in vitro in the absence of peptide. The T11D3-Qdm folding product was observed to bind labeled Qdm peptide, yet the resulting complexes appeared to be unstable (Fig. 5). These results leave the unanswered question of whether T11 normally assembles with peptides in cells and, if so, would it be relevant to determine the nature of the peptides and the extent to which the peptide-loading specificity differs from T23. In addition, there is limited information on the nature of peptides other than Qdm that can be presented by T23 (Qa-1). To directly address these questions, T11D3- and T23D3-bound peptides were eluted from Hela-T11D3 and Hela-T23D3 cells and identified by LC-MS/MS. Background peptides identified in eluates from control Hela-MigR1 cells were subtracted from the eluted peptide pools.

Acquired MS/MS spectra from peptide pools eluted from T23D3 were initially searched using Spectrum Mill Proteomics Workbench (Agilent Technologies), yielding 190 peptide sequences. These were further culled through a stringent validation process, involving expert manual inspection of the LC-MS/MS fragmentation spectra as previously described (51), resulting in 82 peptides with 41 unique sequences (Table I). The two HLA class Ia leader peptide-derived sequences VMAPRTLIL and VMAPRTLVL from HLA-C and HLA-A together represented ∼11% of the validated peptide hits. These well-established ligands for HLA-E and CD94/NKG2A in humans have previously been shown to bind Qa-1 with affinities similar to Qdm. This result is consistent with the dominant presentation of Qdm (or related peptides) in TAP-expressing cells. However, it is noteworthy that a large variety of other peptides were also identified. Peptides ranged in length from 8–12 aa, with a strong predominance of 9-mers (Fig. 7A). The T23D3-bound peptides shared a clear motif, with strong preference for leucine at P9, as well as a preference for alanine at P2 (Fig. 7C). Proline is prominent at P4, and this amino acid is conserved in the mouse and human class Ia leader peptides recognized by CD94/NKG2A receptors. Hydrophobic amino acids are present in the C-terminal position in all of the peptides, regardless of length, with similar representation of amino acids as shown for 9-mers. The peptides identified are derived from proteins with broad intracellular distribution, including slightly >50% from the cytoplasm and/or nucleus, and the remainder with predominant localization in plasma member, ER, Golgi, or mitochondria. The two class Ia leader sequence-derived peptides were the only peptides identified from ER leader sequences. These results indicate that even in TAP-expressing cells, Qa-1b can load with a diverse repertoire of peptide sequences sharing a common motif.

Eighty-nine peptide sequences were identified on initial analysis of samples from T11D3, with 32 confirmed by manual validation, including 23 unique sequences (Table I). The length distribution was similar to that observed with T23D3, with dominant representation of 9-mers (Fig. 7A). There was a striking degree of overlap in peptides isolated from T11 and T23 (Fig. 7B), including

FIGURE 5. Qdm binding capability of T11D3. (A) Eu-based immunoassay to test the ability of folded T11 and T23 monomers of binding Qdm peptide. Folded MHC class Ib monomers were captured on plates by the coated the anti-β2m mAb and incubated with the biotin-labeled peptides at room temperature overnight. The assay was set up in triplicates (n = 3), and the experiment was repeated twice with similar results. One of them is shown. (B and C) FP assay. Folded MHC class Ib monomers were incubated with Alexa Fluor 488–labeled Qdm peptides, and the FP signal was recorded every 60 s at 37 or 25˚C, respectively. The experiments were repeated twice (37˚C) and three times (25˚C) with similar results. One of each is shown.
17 sequences shared between the two samples (Table I). The motif identified with peptides eluted from T11 is very similar to that obtained with T23 (Fig. 7C, 7D), and there was no discernable difference in the subcellular localization of the source proteins. In contrast to T23, class Ia leader peptides were not as prominent among the validated peptides, with only one hit representing 3% of the total peptides. These results provide direct evidence that T11 normally assembles with peptides, and it shares a peptide-binding motif very similar to that of Qa-1.

Discussion

A large number of MHC class Ib genes remain to be characterized with respect to expression and potential function. Analysis is challenging because of the high level of sequence homology among class I genes and the limited availability of specific mAbs. In the current study, we investigated the potential for expression and function of the H2-T11 gene from C57BL/6 mice, a gene with a high degree of homology to the Qa-1b–encoding H2-T23 gene. After cloning and sequencing the T11 gene, RT-PCR was used to demonstrate that correctly spliced T11 mRNA is expressed widely in tissues and leukocyte subpopulations and that expression is particularly high in thymus. Expression was also prominent in spleen, intestine, lung, and liver. The highest expression of T11 was observed in the DP subpopulation of the thymocytes. The possibility that T11 may have a special function in thymic selection remains to be explored. Previous studies have demonstrated that, in contrast to conventional class Ia–restricted T cells, class Ib–restricted T or NKT cells can be positively selected by thymic hematopoietic cells (including DP thymocytes) expressing Qa-1, H2-M3, or CD1 (31, 62, 63).

Further support for the conclusion that H2-T11 encodes a functional MHC protein was obtained using cDNA encoding a chimeric T11 protein with a substituted \( \alpha_3 \) domain that allowed detection by an existing mAb. The chimeric T11D3 protein was expressed at high levels on the surface of both TAP-expressing Hela and TAP-deficient T2 cells, at levels very similar to those observed with the control T23D3 cDNA. Confirmation that the natural T11 protein is assembled and expressed on the surface of primary tissues will require the generation of an appropriate mAb. Our study does not exclude the possibility that the T11 \( \alpha_3 \) domain contains substitutions that interfere with protein assembly. T11 differs from the known Qa-1 alleles at four amino acid positions in the N-terminal segment of the \( \alpha_3 \) domain (residues 191, 195, 197, and 198). However, the amino acid present at each of these positions in T11 is also present in other mouse class I molecules. For example, 191R is present in H-2Kk, and 195P/197G/198D are present in H-2Kd and H-2Dd. In addition, these residues are largely surface exposed. Thus, it is likely that the T11 \( \alpha_3 \) domain is functional and that the T11 protein is expressed at the surface of cells in tissues.

Given the high degree of sequence similarity between T11 and Qa-1, we were interested in determining whether Qa-1 function is conserved in T11. In addition to its function in regulating NK cell activation, Qa-1 has been demonstrated to function in T cell Ag presentation. Control experiments demonstrated that Hela cells expressing chimeric T23D3 molecules were highly functional in presenting Ag to insulin-specific, Qa-1b–restricted 6C5 T cells. By contrast, no responses were observed in Ag-presentation experiments with cells expressing T11D3, indicating the T11 cannot substitute for Qa-1 in Ag presentation to 6C5 T cells. In addition, two substitutions in the \( \beta \)-sheet floor of the peptide-binding groove, T11 has six substitutions in surface-exposed positions of the \( \alpha_1\alpha_2 \) helices as compared with Qa-1\(^\beta\). Any of these substitutions could impact TCR recognition. Although it is possible that some T cells may...
cross-recognize Qa-1 and T11, there are enough amino acid substitutions in the α1a domain to make it unlikely that T cell cross-recognition is common. Qa-1 plays a major role as the exclusive ligand for CD94/NKG2 inhibitory and activating receptors on NK cells. Receptor recognition is highly specific for the sequence of the Qa-1–bound peptide. Thus, it was important to determine whether the capacity to bind to the canonical class Ia leader sequence–derived peptide Qdm is conserved in T11. Recombinant T11D3 H chain was observed to fold in vitro in the presence of Qdm peptide and β2m with an efficiency similar to that observed with the control T23D3 H chain. The capacity of T11 to bind Qdm was further confirmed in peptide exchange reactions with folded T11D3-Qdm protein and labeled Qdm peptide. The possibility that T11 can serve as an alternative ligand for CD94/NKG2 receptors was investigated using MHC tetramers and flow cytometry. Control T23D3-Qdm tetramers were observed to stain large populations of NK cells and NKT cells from spleen and liver, demonstrating that α3 domain substitution does not prevent binding of Qa-1 to CD94/NKG2 receptors. By contrast, no staining was observed with comparable T11D3-Qdm tetramers, supporting the conclusion that T11 cannot serve as an alternative ligand for CD94/NKG2.

The cocrystal structure of HLA-E bound to human CD94/NKG2A demonstrates that the inhibitory receptor binds to the

### Table I. Peptide eluted from T23 and T11

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same general surface of the MHC molecule as do TCRs (64). Examination of this structure suggests T11 position 65 as a candidate receptor contact residue that might preclude recognition by CD94/NKG2A (57). This position contains a nonconservative arginine substitution in T11 replacing tryptophan in all Qa-1 alleles. It is noteworthy that, like T11 and unlike Qa-1, arginine is also present at this position in HL-A-E. It has been previously demonstrated that although peptide-binding specificity is conserved between Qa-1 and HL-A-E, CD94/NKG2 receptors do not cross-recognize species-mismatched MHC class I b molecules, even if they are bound to a species-matched leader peptide (65). Thus, there has been coevolution of CD94/NKG2, its MHC class I b ligand, and the MHC class I a leader sequences that provide the capacity to bind Qdm and related peptides.

Our results demonstrate that recombinant T11D3 has the capacity to bind Qdm peptide. Size-exclusion chromatography, immunoprecipitation, and far-UV CD analysis indicated that recombinant T11D3, generated in vitro by the folding of H chain with β2m and Qdm, assembles similarly to conventional MHC class I molecules. In addition, this protein can bind labeled Qdm through a peptide-exchange reaction. However, an atypical thermal denaturation profile was observed in CD measurements with recombinant T11D3-Qdm consistent with entry into a relatively stable misfolded conformation with preservation of some β-sheet secondary structure at increasing temperatures. In addition, the kinetics of unfolding and refolding of recombinant T11D3-Qdm were determined by size-exclusion chromatography and far-UV CD analysis.

FIGURE 7. Peptide elution from Hela-T11D3 and Hela-T23D3 cells. (A) Length distribution of the peptides eluted from Hela-T11D3 and Hela-T23D3 cells. (B) The unique peptides from the T23- and T11-eluted peptide pool were analyzed. Numbers of 8-, 9-, and 10-mer peptides are shown in the Venn diagram. The results suggested that T11 loads with a diversity of predominantly 9-mer peptides that share a common motif, with dominant preference for aliphatic and hydrophobic amino acids at P9 and preference for alanine at P2. This motif is very similar if not identical to that obtained with T23D3. Indeed, a large majority of the peptide sequences identified from T11D3 were also identified in the T23D3 peptide sample. T11 differs from Qa-1β in only two positions inside the peptide-binding groove, T9H and A11V. These residues are located in the floor of the peptide-binding groove, interacting primarily with the α1 helix but not with bound peptide. A11V is a conservative substitution, and 9H is present in the a, c, and d alleles of Qa-1 that, like the b allele, bind Qdm and serve as functional ligands for CD94/NKG2 receptors. Based on the crystal structure of Qa-1β-Qdm, none of the amino acids that differ between T11β and all Qa-1 alleles directly contact peptide (57). Thus, it appears that the peptide-binding specificity of Qa-1 is conserved in T11.

Early work demonstrated that Qa-1 predominantly loads with Qdm, in striking contrast to that large diversity of peptides that assemble with class I a molecules (21). Crystal structures of Qa-1 and its human ortholog HLA-E with bound leader peptides have demonstrated five primarily hydrophobic anchor sites distributed throughout the length of the groove that accommodate side chains from peptide positions P2, P3, P6, P7, and P9, with P2, P7, and P9 being relatively deep (57, 66). This distribution of pockets may in part account for the exceptionally restricted peptide-binding specificity of these MHC molecules, favoring sequences closely related to Qdm. It is also important to note that, despite having ideal sequences for binding Qa-1 and HLA-E, the Qdm-related peptides dissociate relatively rapidly from the MHC molecules (25). Thus, only a relatively small fraction of Qa-1-binding peptides with near optimal affinities may form complexes that survive long enough to be present in appreciable quantities at steady state.

An important finding in the current study is the identification of a relatively large number of different Qa-1 (T23D3)–bound peptides. Qdm-related HL-A-C– and HL-A-A–derived leader sequences were readily detected in the eluted peptide samples, representing ~11% of the identified peptides, yet many other peptide sequences were also present. A number of studies have characterized T cells with specificity for alternative peptide Ags presented by Qa-1, demonstrating that Qa-1 can bind and present peptides other than Qdm. An Hsp60-derived peptide was identified by mass spectrometry from Qa-1 isolated from cells lacking a source of Qdm (55). In the current study, the peptides isolated from T23D3 were predominantly 9-mers, and a clear motif was identified, with dominant preference for hydrophobic amino acids at P9 and preference for alanine at P2 and P3. Some degree of selectivity was also observed at the other anchor positions (P6 and P7). The anchor positions in Qa-1 have been defined by binding experiments with substituted peptides (24) and from the Qa-1β–Qdm crystal structure (57). The results are generally consistent with findings from a previous study analyzing the relative preference for specific amino acids at each anchor position based on in vitro folding reactions with pools of substituted Qdm peptides randomized at individual positions (24). In the latter study, the greatest observed specificity was for leucine at P9. Previously described Qa-1–restricted T cell epitopes, as well as the Qdm-related leader peptides from mice and humans, contain leucine at P9 and hydrophobic residues at P2. These include the ERAAP-sensitive self-peptide FL9 (FYAEATPML) (38), a Salmonella peptide (GMQFDRGYL) (27), peptides from proinsulin (ALWMRFLPL) (29), influenza (FYAEATPML) (24), and an epitope recognized by guest on April 21, 2017 http://www.jimmunol.org/ Downloaded from LC-MS/MS analysis of peptides eluted from T11D3 isolated from transduced Hela cells demonstrated that T11 loads with a diversity of predominantly 9-mer peptides that share a common motif, with dominant preference for aliphatic and hydrophobic amino acids at P9 and preference for alanine at P2. This motif is very similar if not identical to that obtained with T23D3. Indeed, a large majority of the peptide sequences identified from T11D3 were also identified in the T23D3 peptide sample. T11 differs from Qa-1β in only two positions inside the peptide-binding groove, T9H and A11V. These residues are located in the floor of the peptide-binding groove, interacting primarily with the α1 helix but not with bound peptide. A11V is a conservative substitution, and 9H is present in the a, c, and d alleles of Qa-1 that, like the b allele, bind Qdm and serve as functional ligands for CD94/NKG2 receptors. Based on the crystal structure of Qa-1β-Qdm, none of the amino acids that differ between T11β and all Qa-1 alleles directly contact peptide (57). Thus, it appears that the peptide-binding specificity of Qa-1 is conserved in T11.
by T cells with specificity for TAP-deficient tumor cells (FAPLPRPLPTL) (37).

Recently, Oliveira et al. (37) reported a large number of peptides identified by LC-MS/MS isolated from TAP-deficient EC7.1 cells expressing a chimeric Qa-1β1 protein containing the H-2Dβ-α3-domain, analogous to the constructs used in our experiments. The distribution of peptide lengths was greater as compared with our results. The frequent presence of Leu at the C terminus as well as Ala at P2 was consistent with our current results with TAP-distribution of peptide lengths was greater as compared with our domain, analogous to the constructs used in our experiments. The showed no specificity at the P5 position). As noted above, P5 is present in their peptides but absent from our results (which

expressing cells. By contrast, a very strong signature for Asn at P5 was present in their peptides but absent from our results (which shared by Qa-1b and T11b. It appears very likely that T11 is a conformation of the peptide, favoring optimal positioning of anchor ci

References