A Naturally Processed Mitochondrial Self-Peptide in Complex with Thymic MHC Molecules Functions as a Selecting Ligand for a Viral-specific T Cell Receptor

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Abstract

Peptide fragments of self-proteins bound to major histocompatibility complex molecules within the thymus are important for positively selecting T cell receptor (TCR)-bearing $CD4^+CD8^+$ double positive (DP) thymocytes for further maturation. The relationship between naturally processed thymic self-peptides and TCR-specific cognate peptides is unknown. Here we employ HPLC purification of peptides released from H-$2K^b$ molecules of the C57BL/6 thymus in conjunction with mass spectrometry (MS) and functional profiling to identify a naturally processed K^b-bound peptide positively selecting the N15 TCR specific for the vesicular stomatitis virus octapeptide (VSV8) bound to K^b. The selecting peptide was identified in 1 of 80 HPLC fractions and shown by tandem MS (MS/MS) sequencing to correspond to residues 68–75 of the MLRQ subunit of the widely expressed mitochondrial NADH ubiquinone oxidoreductase ($NUbO_{68–75}$). Of note, the peptide differs at six of its eight residues from the cognate peptide VSV8 and functions as a weak agonist for mature CD8 single positive (SP) N15 T cells, with activity 10,000-fold less than VSV8. In N15 transgenic (tg) recombinase activating gene $2^{-/-}$ transporter associated with antigen processing $1^{-/-}$ fetal thymic organ culture, $NUbO_{68–75}$ induces phenotypic and functional differentiation of N15 TCR bearing CD8 SP thymocytes. Failure of $NUbO_{68–75}$ to support differentiation of a second K^b-restricted TCR indicates that its inductive effects are not general.

Key words: positive selection • thymocyte development • CTL • naturally processed peptides • TAP-$1^{-/-}$

Introduction

TCRs are generated in the thymus through a recombinatorial mechanism involving rearrangement of TCR α and β genes, thereby creating a diverse array of receptor specificities (for a review, see reference 1). Thymocytes bearing TCRs useful to the organism are maintained, whereas those displaying potentially harmful autoreactive specificities are deleted. The process termed positive selection enriches for thymocytes with valuable TCR specificities, i.e., ones recognizing foreign peptides (viral, bacterial, tumor, etc.) bound to self-MHC molecules (2–7). In contrast, negative selection removes autoreactive thymocytes through an apoptotic process (8). Collectively, these two selection processes shape the repertoire of T cells in a given organism.

That T cells preferentially recognize peptides in association with MHC molecules from the thymus within which those same T cells developed was the basis for the idea of positive selection (3, 7). Previously, the role of peptides in the positive selection process was unidentified. However, recent studies of T cell development in animals harboring natural mutations in MHC molecules showed positive selection requires peptides (9–11). This conclusion was independently confirmed by experimental approaches using fetal thymic organ cultures (FTOCs) and MHC-deficient animals (12–15). Positively selecting ligands may be distinct in sequence from the cognate peptides or alternatively, quite similar with only subtle differences at single amino acid positions (16–20). In some studies, only pep-

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tide variants with antagonistic functional activity have been found to be positively selecting (21, 22), while in other examples, the positively selecting peptide has been a weak agonist (16, 20, 23, 24). The latter studies suggest that a given peptide can function as a positively selecting ligand at one concentration and a negatively selecting ligand at a higher concentration (21, 24). Despite this complexity, arising perhaps from variation in TCR transgenes and/or MHC-deficient backgrounds used to examine these processes (transporter associated with antigen processing [TAP]$^{*-/-}$ versus $\beta 2M^{-/-}$), one overwhelming consensus exists: MHC-complexed peptides (pMHC) which enhance affinity for a TCR interaction induce negative selection compared with peptides in complex with the same MHC that show weaker affinity for that TCR. Moreover, those pMHC complexes with faster TCR off-rates may be more favorable at inducing positive selection than those with slower off-rates (25, 26). Presumably, the weaker affinities of positively selecting pMHC ligands trigger survival signals and, unlike the negatively selecting TCR ligands, fail to activate the apoptotic program of the double positive (DP) thymocyte.

Efforts to identify naturally processed self-peptide ligands fostering thymocyte development would aid in the understanding of positive selection. Moreover, such information would help to delineate distinctions between selecting versus antigenic peptides. To date, analysis of natural peptides has used tumor cells or thymic epithelial cell lines as a selecting peptide source; although informative, the nature of thymic peptides must be inferred (17, 18, 27). To identify the number and nature of positively selecting peptidic components within the thymus of a normal animal, this study was conducted using the well-defined N15 TCR transgenic (tg) recombinase activating gene (RAG)-$2^{-/-}$ H-2^b system and C57BL/6 thymic peptides. Here we show that (i) a positively selecting peptide could be identified among 80 pools of peptides eluted from thymic K^b in C57BL/6 mice, (ii) this peptide bears virtually no sequence identity to VSV8 but is an abundant constituent of K^b complexes in the B6 thymus, being derived from the mitochondrial enzyme NADH ubiquinone oxidoreductase ($NUbO_{68–75}$), and (iii) $NUbO_{68–75}$ represents an extremely weak functional agonist for mature N15 TCR-expressing CD8 single positive (SP) peripheral T cells.

Materials and Methods

Mice. N15 TCRtg RAG-$2^{-/-}$ H-2^b, N15 TCR tg RAG-$2^{-/-}$ $\beta_2M^{-/-}$ H-2^b, and N15 TCRtg RAG-$2^{-/-}$ TAP-$1^{-/-}$ H-2^b mice were generated as described previously (28). C57BL/6 TAP-$1^{-/-}$ mice were purchased from Taconic. The lack of RAG-2, β_2M, or TAP-1 gene expression in knockout animals was identified based on the FACS® analysis of peripheral blood cells and Southern blotting or PCR on genomic DNA (28). The homozygosity of the N15 TCR transgenes was proven by subsequent breeding analysis. All lines were maintained and bred under sterile barrier conditions at the animal facility of Dana-Farber Cancer Institute.

Peptide Synthesis. Peptides were synthesized by standard solid phase methods on an Applied Biosystems 430A synthesizer at the Biopolymers Laboratory of Massachusetts Institute of Technology. All peptides were purified by reverse phase HPLC (HPLC 1100; Hewlett Packard) with a C4 2-mm column. Peptides were analyzed for purity and correct molecular weight by electrospray mass spectrometry (MS), amino acid analysis, and HPLC.

Abs and Flow Cytometric Analysis. The following mAbs were used: R-phycoerythrin anti–mouse CD4 (H129.19) and FITC anti–mouse CD8α (53-6.7; BD PharMingen). For flow cytometry, single cell thymocyte suspensions were prepared in PBS containing 2% FCS and 0.05% NaN_3. Thymocytes were stained at 5×10^6 cells per milliliter in PBS, 2% FCS and 0.05% NaN_3 containing the Abs at saturating concentrations. Phenotypes and proportions of thymocyte subsets were analyzed by two-color flow cytometry using FACScan™ (Becton Dickinson) and the CELLQuest™ program. Dead cells were excluded from the analysis by forward and side scatter gating.

DP Dulling Assay. Peritoneal exudate cells (PECs) from TAP-$1^{-/-}$ H-2^b mice, induced 5 d previously with 2 ml of 3% thioglycollate, were suspended in AIM-V medium (Life Technologies) containing 50 μM 2-ME and plated at 10^5 per well in a 96-well microtiter plate. After adherence for 2 h, monolayers were washed with AIM-V medium four times. Thymocytes (5×10^5) from 4–6-wk-old N15tg RAG-$2^{-/-}$ $\beta_2M^{-/-}$ H-2^b mice were cocultured with each HPLC purified thymic H-$2K^b$–derived fraction or synthetic peptide plus PEC for 18 h at 37°C, and stained for the expression of CD4 and CD8α.

Extraction of Self-Peptides from K^b Molecules. K^b molecules were immunoprecipitated essentially as described previously (29). In brief, thymi from 50 C57BL/6 mice were lysed with 50 ml of buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP-40, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 10 μg/ml pepstatin, 1 mM EDTA and 0.05% sodium azide, and insoluble material was removed by centrifugation. The cell lysate was incubated overnight with 2.0 ml of Protein A beads covalently coupled with the anti–H-$2K^b$ mAb, Y3, at 5 mg/ml. After eight washes, the immunoprecipitate was eluted by treatment with 5 ml of 0.1 N acetic acid, pH 3.0, and denatured by boiling for 10 min in 10% acetic acid. Low molecular weight material was collected by passage over a 10,000 MW cut-off membrane (Microcon 10; Millipore). After concentration to the volume of 20 μl, the material was resuspended in 150 μl of H_2O containing 0.1% TFA. HPLC fractionation was carried out on a Varian model 9012 instrument, using a Rainin C18 column (Microsorb 100Å, 5 μm, 15 cmL; Varian Chromatography Systems). The initial buffer system consisted of A and B, which were 0.1% TFA in deionized water and 0.1% TFA in acetonitrile, respectively. A linear gradient from 0–80% buffer B was used >40 min. The flow rate was 0.2 milliliter per min and fractions were collected at 0.5 min intervals (100 μl per fraction). 80 μl of the fractionated samples were lyophilized and resuspended in H_2O before biological analyses.

Sequence Determination of Peptides. Sequence information was determined by microcapillary reverse-phase chromatography directly coupled to a Finnigan LCQ quadrupole ion trap mass spectrometer equipped with a custom nanoelectrospray source. The column was packed in-house with 5 cm of C18 support into a

*Abbreviations used in this paper: DP, double positive; FTOC, fetal thymic organ culture; MS, mass spectrometry; MS/MS, tandem MS; PEC, peritoneal exudate cell; pMHC, peptide–MHC complex; RAG, recombinase activating gene; SP, single positive; TAP, transporter associated with antigen processing; tg, transgenic.

New Objective one-piece 75-μm column terminating in an 8.5-μm tip. Flow rate was a nominal 200 nanoliters per min. The ion trap was programmed to acquire successive sets of three scan modes consisting of full scan MS of 395–1,300 m/z, followed by two data dependent scans on the most abundant ion in those full scans. These dependent scans allowed the automatic acquisition of a high resolution (zoom) scan to determine charge state and exact mass, and tandem MS (MS/MS) spectra for peptide sequence information. MS/MS spectra were acquired with a relative collision energy of 30–35%, an isolation width of 2.5 daltons and recurring ions dynamically excluded. De novo sequence interpretation of the resulting MS/MS spectra was facilitated by the program FuzzyIons developed in the Harvard Microchemistry Facility and the SEQUEST algorithm (30, 31).

FTOC. Fetuses of N15tg RAG-$2^{-/-}$ TAP-$1^{-/-}$ H-2^b mice were dissected at day 16.5 (plug = day 1) and fetal thymic lobes were cultured with or without the indicated peptides in AIM-V medium containing 50 μM 2-ME as described previously (20, 32). The medium was replaced every 48 h. After 7 d, thymocytes were stained for the expression of CD4 and CD8α, or were tested for their capacity to respond to antigen in a 2-d proliferation assay, as described below.

Proliferation Assay. Thymocytes from the organ cultures or fresh LN cells from N15tg RAG-$2^{-/-}$ H-2^b mice (10^5 cells per well) were incubated at 37°C with 2×10^4 irradiated EL-4 cells, which were preloaded for 2 h with the indicated doses of peptides in AIM-V medium containing 50 μM 2-ME with or without 100 U/ml rIL-2. After 48 h of incubation, 0.4 μCi per well of ^{3}H-TdR (ICN Biomedicals) were added, and after an additional 18 h of culture at 37°C, the cells were harvested and the incorporated radioactivity was measured.

RMA-S H-2K^b Stabilization Assay. RMA-S cells were incubated for 18 h at 27°C in RPMI 1640 medium containing 10% FCS to induce H-2K^b expression (33). A total of 10^{-12}–10^{-4} M of each peptide was added to 10^6 RMA-S cells in 1 ml final volume of the RPMI 1640 medium containing 1% FCS. After an additional 1 h incubation at 37°C, the cells were moved to a 37°C incubator. After 4 h of incubation at 37°C, cells were washed, subdivided, and stained in parallel with an excess of the anti–H-2K^b mAb, HB 158 (AF6-88.5.3) and Y3, followed by FITC-conjugated goat anti–mouse IgG Ab. Fluorescence of stained cells was determined on a FACScan™ and analyzed with CELLQuest™ software.

Results

Analysis of Naturally Processed, K^b-bound Thymic Self-Peptides. To characterize those endogenous peptides from the C57BL/6 thymus able to positively select a TCR with a foreign peptide specificity, we employed the N15 TCR tg RAG-$2^{-/-}$ H-2^b mouse system. In this mouse, CD8 SP T lymphocytes bearing the TCR of the N15 CTL clone recognize the foreign vesicular stomatitis virus nucleoprotein amino acids 52–59 (VSV8)(RGYVYQGL) bound to the MHC class I molecule, H-2K^b. This viral peptide in complex with H-2K^b is the major determinant against which protective CTLs are generated in the C57BL/6 mouse. On the RAG-$2^{-/-}$ background, N15 is the only TCR expressed, thereby allowing unambiguous analysis of cell fate in the N15 TCR tg RAG-$2^{-/-}$ H-2^b background. For these experiments, K^b molecules were immunoaffinity purified from NP40-lysed thymii of C57BL/6 mice using the anti-K^b mAb Y3. Subsequently, peptides bound to Sepharose-associated K^b molecules were retrieved by acid denaturation followed by passage over a 10,000-MW cut-off membrane to remove nonpeptidic components. The peptides were further separated based on their hydrophobic characteristics by reverse-phase HPLC using a C18 column (Fig. 1 A, top) and an aliquot of each HPLC fraction was tested for its ability to interact with the N15 TCR in biological assays (Fig. 1 A, bottom). Although FTOC has been

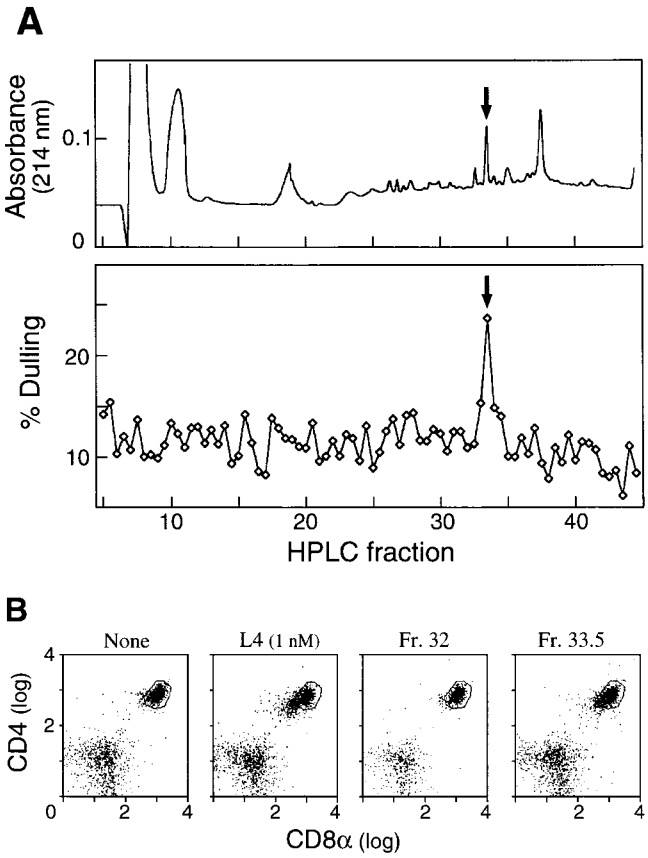

Figure 1. Biochemical fractionation and DP thymocyte dulling bioassay of peptides eluted from C57BL/6 thymic K^b molecules. (A) Detection of an HPLC fraction that promotes DP dulling in N15 tg RAG-$2^{-/-}$ β2M$^{-/-}$ H-2^b thymocytes. Each of 80 HPLC-separated fractions of natural peptides eluted from the H-2K^b molecules from C57BL/6 thymii was tested in a dulling assay. The chromatograph of peptides eluted (shown as 214 nm absorbance U) from H-2K^b molecules of C57BL/6 murine thymii (top) and the DP dulling activity (shown as a percentage of cells with a CD4/CD8 staining lower than controls) (bottom) of each fraction are shown. DP dulling activity is expressed as the percentage of DP cells with a reduction in the expression of CD4 and CD8α, thus resulting in immunofluorescence outside the specified DP thymocyte gate. The percentages of DP cells with CD4/CD8 dulling in cultures with 1 nM L4 peptide, a known weak agonist for the N15 TCR, or no peptides, were 37.5 and 11.4%, respectively. Arrows show a single fraction positive for DP dulling activity. (B) The CD4 versus CD8 staining profile of total thymocytes is shown after treatment with 80% of the material from the positive fraction (fraction 33.5), a representative adjacent negative fraction (fraction 32) or an equivalent volume of 1 nM synthetic L4 peptide. "None" is a control histogram containing thymocytes plus PECs cultured in the absence of any exogenous peptide additions.

used to identify peptides that have the capacity to induce positive selection of thymocytes bearing various TCR transgenes, this assay is not convenient for screening self-peptides extracted from cells or tissues since large amounts of material are required. Thus, to screen complex mixtures of self-peptides, we used a previously described DP thymocyte dulling assay (17) to determine the potential interaction of the TCR expressed on immature thymocytes with peptides complexed with a given MHC molecule on the surface of APCs. For this purpose, N15 tg RAG-$2^{-/-}$ $\beta2M^{-/-}$ thymocytes were cultured in vitro for 18 h with PECs from TAP-$1^{-/-}$ mice preincubated with HPLC-fractionated mixtures of purified self-peptides. In this assay, TCR interaction with pMHC ligands is detected as a reduction of the intensity of CD4 and CD8 expression on the surface of DP thymocytes.

As shown in Fig. 1 B, dulling is observed with a positive synthetic peptide control, termed L4 (RGYLYQGL), a weak agonistic altered peptide ligand of the cognate VSV8 peptide (16, 20). The percentage of DP thymocytes decreases from 46.8 to 29.9 after treatment with 1 nM L4 relative to the no peptide addition control. Although not shown, with an unrelated K^b-binding peptide SEV9, DP dulling activity is not observed at any peptide concentration tested despite its binding to K^b with affinity comparable to L4 or VSV8 (Fig. 3 C). Thus, the potency of the dulling effect depends upon specific TCR recognition, varying with individual peptides as reported previously (17). Using this assay, we screened 80 HPLC fractions derived from the C57BL/6 thymic K^b-bound peptide mixture. As shown by the functional dulling activity profiled in Fig. 1 A (bottom), a single fraction was identified as positive for DP dulling activity. The subtle but significant reduction in the intensity of the CD4 and CD8 expression on the surface of the N15 tg RAG-$2^{-/-}$ $\beta2M^{-/-}$ DP thymocytes observed after exposure to HPLC fraction 33.5 is seen in Fig. 1 B. After addition of other fractions of which fraction 32 is representative, no significant dulling was observed over the baseline "no peptide" addition. These findings suggest there are a finite number of naturally processed positively selecting peptides within the thymus for the N15 TCR. Consistent with this notion, we failed to identify any peptides eluting from the K^b molecules of the EL-4 tumor cell line active in this assay, aside from the cognate VSV8 peptide spiked into the EL-4–derived natural peptide mix (data not shown).

Identification of a Mitochondrial Enzyme Component as a Source of Self-Peptide. To identify the peptide responsible for the fraction 33.5 dulling activity, an aliquot of the pool (20%) was subject to sequencing by the ion trap MS/MS. A sequence from a doubly charged precursor of m/z = 469.3 was determined to be VNVDYS[K/Q][L/I]. Note the isobaric possibilities at each of the COOH-terminal p7 and p8 residues preclude unambiguous assignment at those two positions. However, SEQUEST analysis (31, 34) identified a match with a known murine protein, NADH ubiquinone oxidoreductase MLRQ subunit at residues 68–75 ($NUbO_{68–75}$) (VNVDYSKL). NUbO is a nuclear-encoded mitochondrial protein component of complex 1 of the NADH ubiquinone complex and is widely expressed in various tissues (35). Importantly, the sequence contains a characteristic hydrophilic K^b anchor residue at p3 (V), p5 (Y), and p8 (L). To verify that the spectrum of the peptide in fraction 33.5 matched that of VNVDYSKL, a synthetic peptide corresponding to the deduced sequence was synthesized and its MS/MS spectrum compared. As shown in Fig. 2, the two spectra are indistinguishable, providing strong evidence for the identity of the fraction 33.5 peptide.

The $NUbO_{68–75}/K^b$ Complex Interacts with the N15 TCR on Immature Thymocytes and Mature T Cells. To next test the functional activity of the constituent identified by MS, the synthetic $NUbO_{68–75}$ peptide was tested in DP dulling assays of immature N15 TCR-bearing thymocytes. As shown in Fig. 3 A, the $NUbO_{68–75}$ peptide induces significant DP dulling activity, reducing the percentages of DP

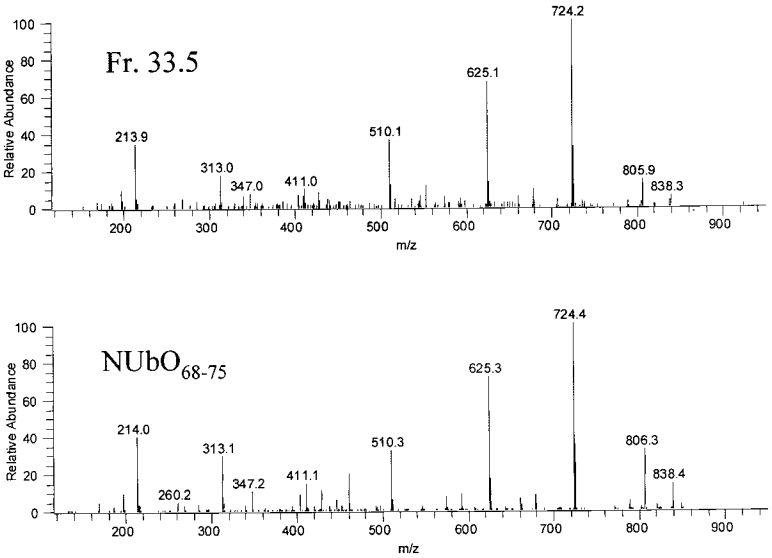

Figure 2. Identification of the peptide sequence from the HPLC fraction positive for the DP dulling activity. Comparison of the MS/MS spectra from fraction 33.5 with that of a synthetic peptide of the sequence $NUbO_{68–75}$. Relative collision energy was 30 and 35%, respectively.

thymocytes from 73 to 16%, 34 and 61% at 10 μM, 1 μM and 100 nM peptide concentrations, respectively. The activity in fraction 33.5 corresponds to a $NUbO_{68–75}$ concentration ≤100 nM (compare Figs. 3 A and 1 B). To examine the ability of $NUbO_{68–75}$ peptide to stimulate proliferation of mature CD8 N15 TCR-bearing T cells, LN cells from N15 tg RAG-$2^{-/-}$ H-2^b mice were cultured with varying molar concentrations of the synthetic $NUbO_{68–75}$ peptide using irradiated EL-4 cells as K^b-bearing APCs. After 48 h of stimulation, cells were pulsed with ^{3}H-TdR and the mean incorporation of duplicate cultures determined. As shown in Fig. 3 B, the cognate peptide VSV8 maximally stimulated ^{3}H-TdR incorporation at ~100 pM-1 nM. In contrast, the $NUbO_{68–75}$ peptide requires a 10-μM peptide concentration to stimulate maximal proliferation. Thus, the identified $NUbO_{68–75}$ peptide is a weak agonist on mature peripheral N15 T cells, differing by ≥10,000 fold from VSV8. This level of agonist activity is comparable to L4 and clearly detectable, unlike with OVAp where no stimulating activity is observed even at a peptide concentration of 0.1 mM. The weak agonist activity of $NUbO_{68–75}$ is not a consequence of poor K^b binding as shown by the RMA-S binding assay (Fig. 3 C). The concentration of $NUbO_{68–75}$ required to yield half-maximal K^b surface expression is less than that of VSV8.

Thymic Selection Mediated by $NUbO_{68–75}$. While the above dulling assay offers a sensitive means to detect TCR–pMHC interaction involving thymocytes and APCs, it does not provide direct information about the ability of the peptides to mediate positive versus negative selection. To ascertain such activity, both in vivo and in vitro assays were performed. Individual N15 tg RAG-$2^{-/-}$ H-2^b mice were injected intravenously with 20 μg of VSV8 or $NUbO_{68–75}$ peptide and the surviving subset of thymocytes examined after 24 h. As shown in Fig. 4, the majority of DP thymocytes are deleted after injection of the VSV8 cognate peptide. Previous studies showed that this deletion involves a caspase-dependent apoptotic mechanism (32). However, $NUbO_{68–75}$ peptide induced no detectable deletion in the DP thymocyte subset in these mice.

To further investigate whether $NUbO_{68–75}$ might induce positive selection of DP thymocytes, we performed FTOC analysis using N15 tg RAG-$2^{-/-}$ TAP-$1^{-/-}$ thymus lobes cultured in synthetic media with or without the $NUbO_{68–75}$ peptide. For comparison, parallel cultures of fetal thymic lobes from N15 tg RAG-$2^{-/-}$ TAP-$1^{+/+}$ were performed. As shown in Fig. 5 A, in the normal H-2^b MHC background, N15 tg RAG-$2^{-/-}$ TAP-$1^{+/+}$, 19% of thymocytes mature into CD8 SP cells whereas in the MHC class I–deficient FTOC, N15 tg RAG-$2^{-/-}$ TAP-$1^{-/-}$, this percent-

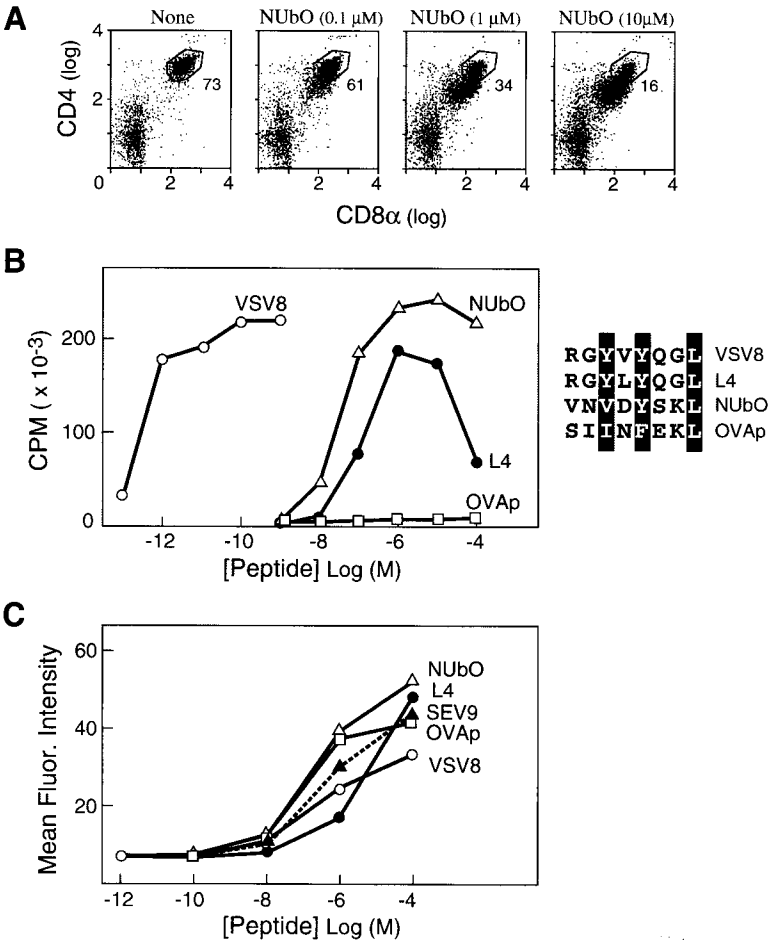

Figure 3. $NUbO_{68–75}$ peptide interacts with N15 TCRs on immature thymocytes and peripheral mature T cells. (A) N15 tg RAG-$2^{-/-}$ β2M$^{-/-}$ H-2^b thymocytes were cultured for 18 h with PECs from TAP-$1^{-/-}$ mice in the presence of the various concentrations of $NUbO_{68–75}$ peptide. Alterations in the expression of CD4 (y axis) and CD8α (x axis) on DP thymocytes were detected by flow cytometry after gating on 10,000 live cells. The percentage of cells in each DP gate is indicated. "None" is a control dot plot containing thymocytes plus PEC cultured in the absence of any exogenous peptide. (B) Proliferation assay of LN cells from N15 tg RAG-$2^{-/-}$ H-2^b mice cultured with various concentrations of the indicated peptides and irradiated K^b-bearing EL-4 cells. Mean of duplicate samples is shown. (C) RMA-S immunofluorescence assay using the K^b-specific mAb HB 158 and indicated peptides at specific molar concentrations. Comparable results were observed with the Y3 mAb as well (data not shown).

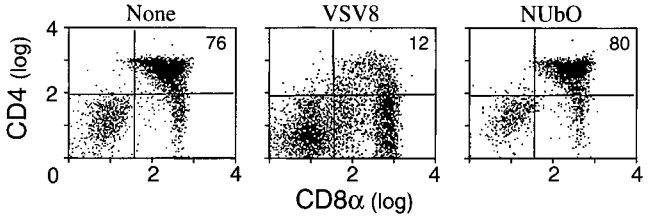

Figure 4. Peptide injection of in vivo VSV8 but not $NUbO_{68–75}$ induces negative selection in DP thymocytes from N15 RAG-$2^{-/-}$ H-2^b mice. 20 μg of VSV8 or $NUbO_{68–75}$ were injected into the tail vein of 3-wk-old N15 tg RAG-$2^{-/-}$ H-2^b mice. 24 h after peptide injection, the expression of CD4 (y axis) and CD8α (x axis) in thymocytes was detected by two-color flow cytometry gating on live cells. The percentages of remaining DP thymocytes are indicated with "none" referring to injection of PBS.

age is reduced by 50%. With addition of 1 μM $NUbO_{68–75}$, however, the percentage of CD8 SP thymocytes is restored. Moreover, at 10 μM $NUbO_{68–75}$ there is a 5–6-fold increase in the percentage of CD8 SP thymocytes which does not rise further with 100 μM $NUbO_{68–75}$. Perhaps more important, Fig. 5 B demonstrates that the phenotypic increase in SP thymocytes induced by 10 μM $NUbO_{68–75}$ is accompanied by functional maturation. Hence, if FTOC is established in the presence of 10 μM $NUbO_{68–75}$ for 7 d, the subsequent immune response of the harvested thymocytes to the VSV8 peptide or the weak L4 variant agonist is increased as judged by cellular proliferation. Furthermore, although 100 μM $NUbO_{68–75}$ also showed a prominent increase in the percentage of the CD8 SP thymocyte subset (55%), the total cellularity of the thymus is reduced and the increase in CD8 SP thymocyte number is not significant (Fig. 5 A and see legend). Although not shown, propidium iodide staining of thymocytes after 7 d of culture with $NUbO_{68–75}$ showed a significant increase in apoptotic cells in the DP thymocyte subset only at 100 μM. Thus, $NUbO_{68–75}$ induces negative rather than positive selection in N15-bearing immature thymocytes at 100 μM. Consistent with the thymocyte phenotype, little proliferation is observed to VSV8 or L4 by thymocytes harvested from FTOCs after culture with 100 μM $NUbO_{68–75}$ (data not shown). These results suggest that $NUbO_{68–75}$ functions as both a positive and a negative selecting peptide ligand for N15 TCR, depending on the density of pMHC in the thymic environment and consistent with the avidity model (23).

Discussion

To identify a naturally occurring, thymic peptide(s) responsible for thymic selection of the VSV8/K^b-specific N15 TCR, peptides from immunoaffinity purified K^b molecules of 50 C57BL/6 (H-2^b) thymii were eluted, fractionated by reverse-phase HPLC, and tested in dulling assays using N15 tg RAG-$2^{-/-}$ β2M$^{-/-}$ H-2^b thymocytes. A single mitochondrial protein (MLRQ)-derived peptide, $NUbO_{68–75}$, was identified, synthesized, and shown to pos-

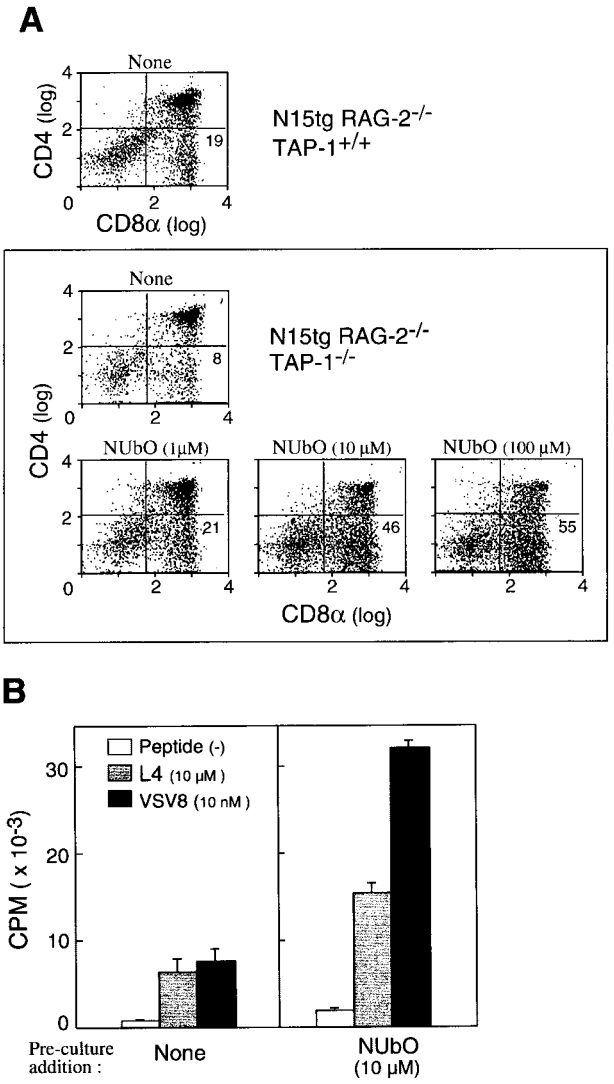

Figure 5. $NUbO_{68–75}$ induces positive selection of N15 tg RAG-$2^{-/-}$ TAP-$1^{-/-}$ H-2^b thymocytes. FTOC was performed by using N15 tg RAG-$2^{-/-}$ TAP-$1^{-/-}$ H-2^b thymic lobes in AIM-V medium with 1, 10, or 100 μM $NUbO_{68–75}$. For comparison, FTOC was performed with N15 tg RAG-$2^{-/-}$ TAP-$1^{+/+}$ H-2^b as well. After 7 d, thymocytes were released from the lobes by passing through a steel mesh and cell numbers were counted. (A) The CD4 (y axis) versus CD8α (x axis) staining profiles of total thymocytes after FTOC are shown for the indicated culture conditions with the percentage of CD8 SP thymocytes indicated. The absolute numbers (mean ± SD) of CD8 SP thymocytes after FTOC of N15 tg RAG-$2^{-/-}$ TAP-$1^{-/-}$ H-2^b with 100 μM, 10 μM, 1 μM $NUbO_{68–75}$, or no peptide were: $0.87 \pm 0.37 \times 10^5$ ($n = 7$); $1.81 \pm 0.32 \times 10^5$ ($n = 6$); $0.83 \pm 0.09 \times 10^5$ ($n = 4$); or $0.69 \pm 0.08 \times 10^5$ ($n = 6$); respectively. The absolute number of CD8 SP thymocytes from FTOC of N15 tg RAG-$2^{-/-}$ TAP-$1^{+/+}$ H-2^b was $1.26 \pm 0.23 \times 10^5$ ($n = 12$). Although not shown, TCR expression as judged by the R53 anticlonotype or H57 anti-Cβ mAb reactivity is increased 5–10 fold on CD8 SP thymocytes relative to DP thymocytes (16). (B) Thymocytes selected on $NUbO_{68–75}$ peptide are functionally responsive to VSV8 and L4. Thymocytes from the above organ cultures with 10 μM $NUbO_{68–75}$ were assayed for their proliferative response to 2×10^4 irradiated EL-4 cells in the presence of 10 nM VSV8, 10 μM L4, or no peptide. ^{3}H-TdR incorporation was determined after 48 h. Results are shown as mean ± SD of triplicate cultures.

sess measurable biological activity. The MS/MS fingerprint of this synthetic peptide was identical to the functionally active HPLC thymic peptide fraction. $NUbO_{68–75}$ mediates positive selection of the N15 TCR in N15 RAG-$2^{-/-}$ TAP-$1^{-/-}$ H-2^b FTOC at 1–10 μM concentrations. At the same time, $NUbO_{68–75}$ does not mediate negative selection upon in vivo injection of N15 tg RAG-$2^{-/-}$ H-2^b mice while the VSV8 cognate peptide induces prompt DP thymocyte deletion at the same dose. For mature N15 CD8 SP T cells, $NUbO_{68–75}$ is a 10,000-fold weaker agonist than VSV8 in molar terms, consistent with the notion that positively selecting peptides have substantially poorer affinities than their cognate peptide counterparts or other full agonists (19, 25). In this regard, earlier studies with synthetic peptides demonstrated that VSV8 and I4 are strong agonists for the N15 TCR and induce negative selection while L4 is a weak agonist and induces positive selection (16, 20).

Characterization of $NUbO_{68–75}$ represents the first identification of a naturally processed self-peptide extracted from normal thymus with selecting ability for a TCR. Whether $NUbO_{68–75}$ is bound to K^b molecules on stromal elements, thymocytes, or both is unclear. Notwithstanding, these current data must be interpreted in the context of earlier studies using cell lines as a source for isolating potential selecting peptides. For example, using the OT-1 TCR specific for OVAp-K^b pMHC and K^b-bearing LB27 tumor cells as a source of K^b-bound peptides, Hogquist et al. (17) identified OT-1 TCR dulling activity in 8 out of 100 HPLC fractions. Of several peptides sequenced, one, derived from F-actin capping protein subunit α1 ($CP\alpha1_{92–99}$), showed dulling activity, and mediated positive selection in OT-1 TAP-$1^{-/-}$ H-2^b FTOCs. Likewise, using tg mice expressing the F5 TCR specific for influenza virus 68 $nucleoprotein_{366–374}/D^b$ pMHC or the P14 TCR specific for lymphocytic choreomeningitis virus glycoprotein $peptide_{33–41}/D^b$ pMHC in conjunction with the thymic epithelial cell line 427.1, several positively selecting peptides were identified (18). F5 TCR-bearing thymocytes were positively selected in F5 tg TAP-$1^{-/-}$ FTOC by mouse histone $H2A.1_{76–84}$ or mouse brain protein $E46_{100–108}$ whereas P14 TCR-bearing thymocytes were positively selected in P14 TAP-$1^{-/-}$ FTOC by mouse ribonucleotide reductase $M1_{634–642}$ but not by $H2A.1_{76–84}$ or $E46_{100–108}$. A common feature to each of these positively selecting peptides is their derivation from a widely expressed protein involved in basic cellular functions. For example, $NUbO_{68–75}$ is one of the four multisubunit enzyme complexes comprising the mitochondrial electron transport chain; CPα1 is derived from an F-actin capping protein subunit integral to the cellular cytoskeleton and ribonucleotide reductase M1 is essential for cell division. All of these proteins, including the brain protein E46, are expressed in thymus. In the cellular sources examined, their derivative fragments represent a significant component of the naturally processed peptide pool. Of note, the alloreactive 2C CTL clone recognizes two distinct specificities, P2 Ca/L^d and dEV-8/K^{bm3} (27). The latter pMHC ligand includes the dEV-8 peptide which also derives from mouse MLRQ. In fact, the p8 valine residue of dEV-8 is the p1 residue of $NUbO_{68–75}$, implying that the small (<100 amino acids) MLRQ gene product may be processed endogenously into several MHC class I–binding fragments. The P2 Ca peptide is also a mitochondrial protein derivative. Although thymus-specific selecting peptides may exist in the context of one or another cell type-specific stromal element, these are not apparently the common selecting peptides based on this limited sampling to date of MHC class I–bound peptides. In the case of MHC class II, comparison of endogenous peptides bound to splenic and thymic MHC molecules also supports this view (36).

Another common feature of naturally derived and positively selecting ligands is their extremely remote, even nonexistent, similarity to the cognate antigen for which a given TCR is specific. For example, in the case of the N15 TCR, the VSV8 ligand (RGYVYQGL) bears identity to $NUbO_{68–75}$ (VNVDYSKL) only at two of the three K^b anchor residue positions, p5 and p8. Consequently, the TCR contact residues are all different. Likewise, for the OT-1 TCR, the OVAp ligand (SIINFEKL) bears identity only at p5 and p8 with CPα1 (ISFKFDHL). Similarly, for the P14 TCR, the lymphocytic choreomeningitis virus gp33–41 peptide (KAVYNFATM) differs from ribonucleotide reductase $M1_{634–642}$ (FQIVNPHLL) at all positions except for the p5 anchor residue asparagine. These differences among peptide ligands mediating positive selection versus their cognate counterparts argue strongly that positive thymic selection mandates a different set of ligand recognition requirements, at least in terms of the biophysical binding parameters of TCR–pMHC interaction, than does mature T cell antigenic recognition.

An important feature of the present findings is that despite the disparity between cognate and selecting peptides, very few peptides unrelated to the cognate antigen are positively selecting. Indeed, we observed but 1 out of 80 HPLC thymic peptide fractions to contain positive selecting activity for the N15 TCR. Likewise, dulling activity was detected in only 7–8 out of 100 tumor cell peptide fractions for the OT-1 TCR. Moreover, $NUbO_{68–75}$ is positively selecting for the N15 TCR but not the OT-1 TCR and conversely, the CPα-1 peptide, which is positively selecting for the OT-1, is not positively selecting for N15 (reference 17 and data not shown). Although the sensitivity of the dulling assay may underestimate the number of positively selecting thymic peptides because of their low molarity in the eluted fractions, certainly the number of positively selecting ligands is finite. Given the primary sequence differences between positively selecting and cognate peptides, it is easy to imagine that a number of peptides may be selecting for a single TCR. In the case of the F5 TCR, as noted above, two distinctly different peptides are positively selecting in F5 TAP-$1^{-/-}$ FTOC (18, 19). Hence, a given positively selecting peptide may induce differentiation of multiple TCRs during development while a single antigenic peptide is able to drive activation of a much more limited number of mature T cells. That a single peptide can induce polyclonal CD8 SP thymocyte devel-

opment in non-tg TAP-$1^{-/-}$ FTOC is consistent with this notion (23) as is the ability of a single pMHCII complex to select diverse TCR-bearing CD4 T cells (37–40).

Structural analysis of TCR-pMHC complexes demonstrate that a majority ($\geq$2/3) of atomic contacts occur between the TCR and the MHC (41–43). Hence, it is conceivable that "weak" intrinsic TCR–self-MHC reactivity is the predominant driving force for positive selection. Notwithstanding, such TCR interaction with $\alpha1/\alpha2$ helices of MHC class I or $\alpha1/\beta1$ helices of MHC class II could be modulated by the MHC-bound peptide by interposing side chains that either enhance or diminish TCR interaction with pMHC. The former peptides may result in negative selection and the latter in positive selection. Reduction in affinities of positively selecting K^b-bound peptides relative to their cognate peptide counterparts by removal of the antigen's more optimal p1, p4, and/or p6 contacts, for example, as in the case of the N15 TCR, may allow for creation of such a positively selecting ligand. In some cases, intrinsic contacts between the TCR and MHC alone may be sufficient for selection with no requirement for peptide participation in recognition whatsoever. Perhaps this is the basis for detectable SP thymocyte differentiation in N15 tg RAG-$2^{-/-}$ TAP-$1^{-/-}$ H-2^b FTOC (assuming there is not a TAP-1–independent loading of $NUbO_{68–75}$ or other selecting peptides). Such a peptide-independent selection concept has been espoused in the past (44). However, given the atomic evidence that a local change of the peptide residue can modulate adjacent MHC side chain positions, thereby amplifying focal local alterations (16), and the restricted nature of selecting peptides shown collectively in the above studies, it is easiest to imagine that while TCR–MHC interactions are the driving force for positive selection, peptides of unrelated sequences to the cognate ligand when bound to the groove of an MHC antigen-presenting platform will modulate such events.

Finally, as each unique TCR-expressing thymocyte moves through its microinductive environment, it senses many different peptides bound to MHC molecules on interdigitating thymic stromal elements. Both the qualitative nature (affinity) and the quantitative nature (avidity) of those different pMHC–TCR contacts must be integrated into the overall selection process. Those TCR-bearing thymocytes which are not deleted via negative selection as a result of too high an overall avidity for a self-pMHC complex or self-MHC alone can then be positively selected. A requirement for such a positively selecting peptide is that it modulates binding of the TCR to a self-pMHC complex above a threshold so that TCR-stimulated survival signals can be provided but not so high that TCR-triggered apoptotic deletion occurs. In the TAP-$1^{-/-}$ FTOC setting, even a positively selecting peptide ligand at high concentration may surpass the deletion avidity threshold in the absence of other unrelated self-peptides that would reduce TCR avidity for the same MHC and result in negative selection. Understanding the precise basis of positive selection will allow for repertoire manipulation to enhance immune protection in the future.

We thank Daniel P. Kirby for expert assistance with tandem mass spectrometry sequencing.

This work was supported by National Institutes of Health grant AI45022 to E.L. Reinherz.

Submitted: 22 June 2001
Revised: 2 August 2001
Accepted: 17 August 2001

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