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# Analysis of Qa-1$^b$ Peptide Binding Specificity and the Capacity of CD94/NKG2A to Discriminate between Qa-1–Peptide Complexes

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## Abstract

The major histocompatibility complex class Ib protein, Qa-1$^b$, serves as a ligand for murine CD94/NKG2A natural killer (NK) cell inhibitory receptors. The Qa-1$^b$ peptide-binding site is predominantly occupied by a single nonameric peptide, Qa-1 determinant modifier (Qdm), derived from the leader sequence of H-2D and L molecules. Five anchor residues were identified in this study by measuring the peptide-binding affinities of substituted Qdm peptides in experiments with purified recombinant Qa-1$^b$. A candidate peptide-binding motif was determined by sequence analysis of peptides eluted from Qa-1 that had been folded in the presence of random peptide libraries or pools of Qdm derivatives randomized at specific anchor positions. The results indicate that Qa-1$^b$ can bind a diverse repertoire of peptides but that Qdm has an optimal primary structure for binding Qa-1$^b$. Flow cytometry experiments with Qa-1$^b$ tetramers and NK target cell lysis assays demonstrated that CD94/NKG2A discriminates between Qa-1$^b$ complexes containing peptides with substitutions at nonanchor positions P4, P5, or P8. Our findings suggest that it may be difficult for viruses to generate decoy peptides that mimic Qdm and raise the possibility that competitive replacement of Qdm with other peptides may provide a novel mechanism for activation of NK cells.

Key words: CD94 • NKG2A • Qa-1 • natural killer cell • major histocompatibility complex

## Introduction

Qa-1$^b$ is a nonclassical MHC class I molecule encoded by the *T23* gene in the T region of the H-2 complex in mice (1–4). Early studies demonstrated that Qa-1 can be directly recognized by alloreactive cytolytic CD8$^+$ T cells (5, 6). Like most class Ib molecules, Qa-1 is relatively nonpolymorphic (7, 8). It is expressed in a wide range of tissues (3, 9) but cell-surface expression appears to be low and the cellular half-life is relatively short (4). A series of studies on the specificity of Qa-1–specific alloreactive T cells led to the discovery that a major fraction of Qa-1–restricted CTLs recognize a nine–amino acid peptide (AMAPRTLLL) from the leader sequence of H-2D or L molecules (9–11). This peptide is termed Qa-1 determinant modifier (Qdm).[1] Recognition by a subset of CTLs was sensitive to a polymorphism mapping to H-2D, with Val instead of Ala in the third position in the D$^k$ leader peptide. Presentation of Qdm by Qa-1 was shown to be dependent on the transporter associated with antigen processing (TAP) (10, 11). Subsequent peptide elution studies demonstrated that the predominant peptide associated with Qa-1 on mitogen-activated T cells and transfected fibroblasts was Qdm (12, 13).

Despite evidence that Qa-1 molecules are dominantly loaded with a single peptide, Qdm, several studies suggest that Qa-1 can bind and present other antigens to T cells. Vidovic et al. reported that recognition of the copolymer Glu-50-Tyr-50 by a γ/δ T cell hybridoma was blocked by

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[1]*Abbreviations used in this paper:* β2m, β2-microglobulin; hsp, heat shock protein; Qdm, Qa-1 determinant modifier; TAP, transporter associated with antigen processing.


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anti–Qa-1 Abs (14). Imani and Soloski demonstrated that a tryptic digest of *Mycobacterium bovis* heat shock protein (hsp) 65 could stabilize Qa-1 expression on the surface of transfected cells, suggesting that Qa-1 may be capable of binding an hsp-derived peptide(s) (15). Recent studies have demonstrated that $CD8^+$ CTLs are generated in mice after infection with *Salmonella typhimurium* with specificity for a nine–amino acid epitope in bacterial GroEL and cross-reactivity with a corresponding mouse hsp60 peptide (16, 17). Qa-1–restricted $CD8^+$ T cells with specificity for *Listeria monocytogenes* have also been demonstrated, but the epitope(s) remain to be defined (18, 19). We previously reported the characterization of Qa-$1^b$–restricted T cell hybridomas derived from low-responder H-$2^b$ mice immunized with pork insulin (20). These T cells recognized an insulin B chain determinant generated though a TAP-independent processing pathway. Finally, Qa-1–restricted regulatory $CD8^+$ T cells have been described with the capacity to specifically kill activated $V\beta8^+$ T cells (21, 22). These T cells, induced by vaccination with $V\beta8^+$ T cells or staphylococcal enterotoxin B injection, presumably recognize T cell receptor–derived peptides presented by Qa-1. These various observations raise the possibility that Qa-1 may have a capacity to bind and present a diverse repertoire of peptides. However, the range of peptides that can be presented is uncertain and the Qa-1 peptide–binding motif has not been defined.

Recent studies have shown that Qa-1 has an important function as the dominant and possibly exclusive ligand for murine C-type lectin family NK cell receptors, CD94/NKG2 (23–26). Heterodimers of NKG2 family members with CD94 recognize HLA-E in humans (27–29). Like Qa-1, HLA-E appears to bind predominantly to certain peptides derived from class I leader sequences (30, 31). In addition, these two proteins have Ser at the normally conserved positions 143 and 147 instead of Thr and Trp, respectively, which are found in other class I molecules. Despite these shared features, Qa-1 and HLA-E are not obvious orthologues based on sequence comparison. HLA-E cell surface expression appears to be dependent on TAP function and coexpression of a class I molecule containing an appropriate leader sequence to serve as a source of peptide (31, 32). In contrast, Qa-1 can be expressed in the absence of TAP and cell surface Qa-1 is not further stabilized in TAP-deficient cells by exogenous Qdm peptide (23). This finding is consistent with the idea that Qa-1 may have a capacity to bind peptides other than Qdm through a TAP-independent mechanism. Alternatively, Qa-1 may be expressed without bound peptide; empty Qa-1 molecules could be unusually stable.

The cytoplasmic domains of human and mouse NKG2A contain immunoreceptor tyrosine-based inhibitory motifs, and CD94/NKG2A heterodimers have been shown to inhibit NK cell function in the presence of HLA-E or Qa-1 ligand (23, 27–29, 33). Human NKG2C and NKG2E lack immunoreceptor tyrosine-based inhibitory motifs, and at least CD94/NKG2C has a capacity to activate NK cells through association with the small signaling homodimer, DAP12 (34). Mouse homologues of these activating receptors have been identified, and they share specificity with the CD94/NKG2A inhibitory receptor for Qa-1 (25). NKG2C and NKG2E mRNA is considerably less abundant than that encoding NKG2A in mouse NK cells, but the distribution of activating and inhibitory receptors on NK cell populations remains to be characterized. Several studies suggest that human CD94/NKG2 receptors may have a capacity to discriminate between HLA-E complexes containing different leader sequence–derived peptides (35–37). The molecular basis of specificity for leader peptides and functional implications remain to be characterized. We previously reported that Qa-1 molecules expressed in TAP-deficient cells were unable to inhibit NK cell function unless exogenous Qdm peptide was provided (23). This indicated that Qa-1–bound peptide is required to generate a functional ligand for murine CD94/NKG2A, but the capacity of this receptor to discriminate between different Qa-1–peptide complexes remained to be addressed.

In this study, we describe experiments designed to investigate the peptide-binding motif of Qa-$1^b$. The results suggest that the leader peptide–derived Qdm sequence is ideally suited for binding Qa-$1^b$. However, our findings also suggest that this class Ib molecule has the potential to bind a relatively diverse repertoire of peptides in addition to Qdm. Further experiments demonstrate that CD94/NKG2A discriminates between Qa-1 complexes containing different peptides.

## Materials and Methods

*Qa-$1^b$ Refolding and Tetramer Formation.* Soluble Qa-$1^b$ and human β2-microglobulin (β2m) were purified and refolded with peptide as described (38). In brief, 6 liters of *Escherichia coli*–containing expression vectors were induced with isopropyl β-d–thiogalactopyranoside and the cells were lysed. Inclusion bodies were purified by washing with a Triton X-100 detergent solution, and solubilized in 8 M urea. The soluble Qa-$1^b$ was folded in vitro with β2m and peptide and purified on a Pharmacia S-300 gel filtration column (Amersham Pharmacia Biotech). In folding experiments with random peptide libraries or peptide pools, 200-ml folding reactions included 1 μM Qa-$1^b$ heavy chain, 2 μM β2m, and 30 mg peptide. To make tetramers, refolded Qa-$1^b$ was biotinylated with the BirA enzyme as described (38), purified on a UnoQ ion exchange column (Bio-Rad Laboratories), and tetramerized with streptavidin-allophycocyanin (Molecular Probes, Inc.) in a 4:1 molar ratio.

*Peptides.* Peptides used in binding experiments were synthesized using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry on a multiple peptide synthesizer (Ranin Symphony) and were labeled as indicated through reaction with cysteine thiol groups with a slight excess of 1-biotinamido-4-(4-[maleimidomethyl]cyclohexane-carboxamido)butane (Pierce Chemical Co.). The random and position-degenerated peptide libraries were assembled by Fmoc-based solid-phase synthesis at the 50 μmol per channel scale on a Wang resin using an Advanced ChemTech model 348O multiple peptide synthesizer. Acylation of the resin was affected using a sixfold molar excess of preformed hydroxybenzotriazole esters of all coded Fmoc-protected amino acids (excluding

cysteine) essentially as described previously (39). A 50% molar excess of Fmoc-Val, Fmoc-Arg(Mtr), and Fmoc-Ile over other Fmoc–amino acids was used in order to compensate for their lower acylation rates (39, 40). After cleavage and deprotection (Reagent K, 2 h, 25°C), the peptides were precipitated with diethyl ether (−20°C, 4 h). The precipitate was collected by centrifugation and solubilized in neat TFA, diluted with water, and lyophilized. The lyophilized libraries were obtained in the form of their TFA salts. Quantitative amino acid analysis and $NH_2$-terminal sequence analysis confirmed their expected composition and randomness of their sequence.

*Peptide Binding Assays.* Competition based peptide binding assays were performed as described (41). In brief, refolded recombinant Qa-$1^b$–Qdm-β2m (30 nM) was incubated overnight at room temperature in 30-μl volumes with 0.2 μM biotin-Qdm4C (AMACRTLLL), 0.01% NP-40 (United States Biochemical), 10 mM citrate-phosphate buffer, pH 5.5, a cocktail of protease inhibitors, and a range of concentrations of unlabeled competitor peptide. Samples were pH neutralized and transferred to 96-well plates coated with anti–human β2m Ab (Immunotech), washed, and incubated with 100 ng/ml of europium-labeled streptavidin (Wallac Oy). Detection occurred at 615 nm using a 1230 ARCUS time-resolved fluorometer (LKB Wallac). $IC_{50}$ was determined by calculating the amount of unlabeled peptide required to displace 50% of the binding of biotin-Qdm4C and dividing by the amount of unlabeled Qdm required to do the same.

*Pool Peptide Sequencing.* Qa-$1^b$ complexes were generated by folding in vitro with random peptide libraries or peptide pools and purified as described above. Qa-$1^b$ complexes (500 μg) were concentrated and peptide was eluted by dilution in 0.5 ml 0.1% TFA/10% $CH_3CN$ and incubation for 30 min at 24°C. Eluted peptide was separated from heavy chain and β2m using a centricon 3 concentrator (Amicon, Inc.). Control samples were prepared by mock elution. Edman degradation of the original libraries and of the Qa-$1^b$ bound/eluted peptides was performed in a model Procise cLC peptide sequencer (PE Biosystems) operated in the pulsed liquid mode essentially as described previously (39, 42). The manufacturer's degradation chemistry cycles were used without modification and model 610A software was used to calibrate the instrument, quantify the phenylthiohydantoin amino acids, and compare the chromatograms.

Peptides eluted from Qa-$1^b$ after folding with peptide pools randomized at specific positions were directly analyzed without further manipulation by microbore reversed-phase HPLC consisting of a model 140A solvent delivery system and a model 1000S diode-array UV detector (PE Biosystems) equipped with a 2.3-μl flow cell. The separation was achieved using a Zorbax SB-C18 column (1 × 150 mm, particle diameter ~5 μm, 300 A pore size) equilibrated at 25°C in 0.1% aqueous TFA and developed at a flow-rate of 90 μl/min using a linear gradient of acetonitrile. The column eluate was monitored at 210 and 280 nm and evaluated using the model TurboChrom software (PE Biosystems). The fractions absorbing at 210 nm were manually collected and stored at −20°C for sequence and mass spectrometric analyses. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis was performed using model Protein-TOF or model Reflex-III mass spectrometers (Bruker Daltonics) equipped with 337-nm nitrogen lasers; α-cyano-4–hydroxycinnamic acid (MALDI-Quality; Hewlett-Packard) was used as a matrix. Electrospray ionization triple quadrupole mass spectrometry analysis was performed on a model API-3000 triple quadrupole mass spectrometer (PE Biosystems/Sciex) equipped with MicroIonSpray electrospray source. In the positive mode, the samples were introduced in 50% aqueous methanol containing 0.1% acetic acid.

*Mice, Abs, and Flow Cytometry.* C57BL/10J mice were purchased from The Jackson Laboratory and maintained by the Emory University Division of Animal Resources in specific pathogen-free conditions. C57BL/6 $\beta 2m^{-/-}$ (The Jackson Laboratory), C57BL/6 $K^{b-/-}$, and C57BL/6 $K^{b-/-}D^{b-/-}$ (a gift of F. Lemonnier, Institut Pasteur, Paris, France) were maintained in the University of California at Berkeley Animal Facility. FITC-conjugated anti-CD3ε, PE-conjugated anti-NK1.1, and CyChrome®-conjugated anti-B220 were purchased from BD PharMingen. Splenocytes were treated to lyse red blood cells before staining in PBS/2% fetal bovine serum/0.1% sodium azide with Fc Block™ (BD PharMingen) on ice. Fluorescence was analyzed using a FACSCalibur™ (Becton Dickinson).

*IL-2–cultured NK Cells and Chromium-release Assay.* Splenocytes pooled from 7–15 C57BL/10 mice were depleted of red blood cells, passed though nylon wool, and cultured with 200 ng/ml rIL-2 (Chiron Corp.). The following day, cultured cells were depleted of T cells by incubating with 10 μg/ml anti-CD4 (clone GK1.5) and anti-CD8 (clone 53-6.72), followed by removal of labeled cells with goat anti–rat IgG magnetic beads (PerSeptive Biosystems). The cells were then returned to culture with IL-2. On day 2 of culture, cells were sorted into Qa-$1^b$ tetramer–positive or –negative NK cells ($NK1.1^+CD3^-$) using a FACS Vantage™ cell sorter, returned to IL-2 media, and assayed on day 5. T2 cells expressing Qa-$1^b$ (T237), a gift of Mark Soloski (The Johns Hopkins University Medical School, Baltimore, MD), were used as target cells. Peptides (30 μM) and $^{51}Cr$ were added to target cells overnight at room temperature, and peptide was also included during the standard 4-h killing assay. In some experiments, spleen cells from 12 $Kb^{-/-}$ mice (~10-wk-old) were cultured with IL-2 for 2 d and then stained with CD3-PE (BD PharMingen), NK1.1-biotin, 3g2-FITC (rat anti-NKG2A/C/E IgG2a), and streptavidin-Red670 (Caltag). The CD3-$NK1.1^+3g2^+$cells were sorted to high purity on a Coulter EPICS Elite cell sorter. The sorted population was returned to culture for an additional 2 d in IL-2 before being used as effectors in chromium-release assays. The day before the functional assay, spleen cells from $\beta 2m^{-/-}$, $K^{b-/-}$, and $K^{b-/-}D^{b-/-}$ mice were put into media containing 2.5 μg/ml ConA. Cells were simultaneously treated with or without Qdm peptide at 50 μM final concentration. The day of the assay, the cells labeled for 1 h at 37°C with $^{51}Cr$ (in the presence or absence of Qdm, as appropriate). Live targets were then purified on a Ficoll gradient and washed three times before being used in a chromium-release assay. Qdm peptide was included in the assay for targets that had been loaded with peptide.

## Results and Discussion

*Identification of Anchor Positions in Qdm.* A peptide-binding immunoassay was developed using soluble recombinant Qa-$1^b$. Biotin-labeled Qdm was synthesized using a substituted peptide with cysteine in the P4 position providing a thiol for selective reaction with a biotin maleimide derivative. Biotin-Qdm4C peptide was incubated with Qa-1 followed by capture in wells coated with anti-β2m. Bound biotin-peptide was detected with europium-streptavidin (41). Binding, which occurs through a peptide exchange mechanism, was saturable and specifically inhibited in the

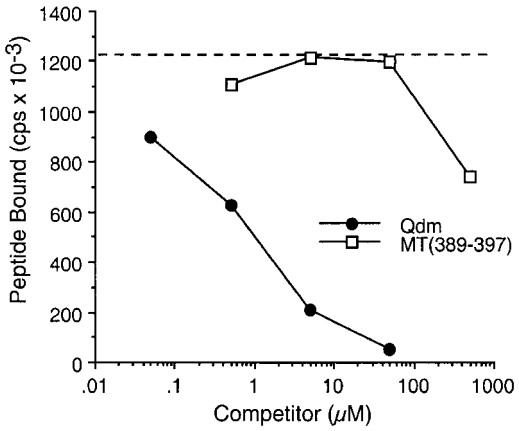


**Figure 1.** Peptide binding to Qa-1$^b$. Soluble recombinant Qa-1$^b$ (30 nM) was incubated overnight at room temperature in duplicate with 0.2 μM biotin-Qdm4C and the indicated concentrations of unlabeled competitor peptide. Qa-1$^b$ was then captured with anti-β2m Ab and bound biotinylated peptide was detected with europium-streptavidin fluorescence, indicated as counts per second (cps). The dashed line indicates quantity of bound biotin-Qdm4C in the absence of competitor peptide. The polyoma virus peptide MT(389–397), RRLGRTLLL, binds Dk with high affinity (reference 41).

presence of excess Qdm competitor peptide but not a control peptide (Fig. 1). Qdm4C was observed to bind to Qa-1 with affinity similar or identical to Qdm in competition assays. No binding was observed with a peptide labeled through a cysteine at P3 (data not shown). Competition assays were used to evaluate the relative affinities of a panel of Qdm derivatives with Lys substitutions at each position. The concentration of competitor peptide required to inhibit 50% of the binding of biotin-Qdm4C was normalized relative to Qdm. Substitutions at P2, P3, P6, and P9 reduced competitive binding activity below detectable levels, indicating that these positions represent anchor residues (Table I). A major reduction in binding was also observed with lysine substitution at P7, whereas substitution at other positions had little effect on binding affinity.

P9 and P2 were previously identified as dominant anchor residues through analysis of the capacity of Ala-substituted Qdm peptides to inhibit binding of labeled Qdm to cell surface Qa-1$^b$ (43). Relatively small effects on binding were observed with Ala substitutions at P3, P6, or P7 in that study (43). This difference probably can be attributed to the nature of the substitutions used in this study, in which smaller nonpolar or polar side chains were replaced by charged Lys rather than Ala. In addition, Kurepa and Forman (43) observed that replacement of Arg with Ala at P5 significantly reduced binding whereas the conservative Lys substitution in this study had little effect. It is notable that the crystal structure of a prototypic HLA class Ia leader peptide bound to HLA-E demonstrated anchor residues at the same five positions (P2, P3, P6, P7, and P9) identified in this study with Qa-1 (44). The P5 Arg side chain was observed in two conformations in the crystal structure, fully solvent exposed or held down by a salt bridge to a residue on the top of the α2 helix (44).

**Table I.** *Competition for Binding of Biotin-Qdm4C to Qa-1$^b$ by Qdm Analogues*

| Competitor peptide | Peptide sequence | $IC_{50}$ |
|---|---|---|
| **Qdm** | AMAPRTLLL | 1 |
| Qdm P1 | KMAPRTLLL | 8 |
| Qdm P2 | AKAPRTLLL | >1,000 |
| Qdm P3 | AMKPRTLLL | >1,000 |
| Qdm P4 | AMAKRTLLL | 6 |
| Qdm P5 | AMAPKTLLL | 5 |
| Qdm P6 | AMAPRKLLL | >1,000 |
| Qdm P7 | AMAPRTKLL | 200 |
| Qdm P8 | AMAPRTLKL | 2 |
| Qdm P9 | AMAPRTLLK | >1,000 |
| Qdm P2T | ATAPRTLLL | 73 |
| Qdm P2L | ALAPRTLLL | 2 |
| Dk signal | AMVPRTLLL | 2 |
| Qa-1 signal | LFAHLLQLL | 115 |
| CD1.1 signal | YLPWLLLWA | >300 |
| Influenza NP | FLARSALIL | 9 |
| hCMV UL40 | VMAPRTLIL | 1 |

NP, nucleoprotein; h, human.

*Analysis of the Qa-1$^b$ Peptide-binding Motif.* Qa-1 was folded in vitro with a fully random nonameric peptide library containing all natural amino acids except Cys as an initial approach to analyze the peptide binding motif (45–47). The acid-eluted peptide pool was subjected to Edman sequencing, and residues with a yield of at least 150% compared with the previous cycle were considered significant and underlined in Fig. 2. A strong signal for Met and Leu but not other amino acids was observed in cycle 2. This is consistent with the assignment of Met at P2 in Qdm as a dominant anchor. A Qdm derivative with Leu at P2 was observed to bind Qa-1 with affinity similar to Qdm (Table I), confirming that Leu can serve as an alternative P2 anchor. The P3 anchor appeared to be rather promiscuous with enrichment for seven different residues. Of the five anchor positions identified above from binding studies with substituted Qdm peptides, the residues found in the Qdm sequence were only identified as anchors at P2, P6, and P7 from pool sequence analysis. Enrichment for selected amino acids was also observed at each nonanchor position. Among these, the Qdm residue Arg was enriched at P5. The significance of preference for specific residues at nonanchor positions remains uncertain. However, preferred residues at nonanchor positions are commonly observed in this type of study (45–48). The absence of enrichment for Leu at P9 (Fig. 2) was of concern because this was identified as the dominant anchor by Kurepa and Forman

| Cycle | A | D | E | F | G | H | I | K | L | M | N | P | Q | R | S | T | V | W | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 103.1 | 29.8 | 21.7 | 44.8 | 101.9 | 17.5 | 90.8 | 13.5 | 17.7 | 39.3 | 17.8 | 4.1 | 22.8 | 67.2 | 22.7 | 12.5 | 71.8 | 5.0 | 45.9 |
| 2 | 22.0 | 7.5 | 10.4 | 5.0 | 27.9 | 19.8 | 81.7 | 4.3 | 131.9 | 165.6 | 8.5 | 4.2 | 26.9 | 39.2 | 5.3 | 4.3 | 28.0 | 3.2 | 39.3 |
| 3 | 32.8 | 16.4 | 19.6 | 11.0 | 15.8 | 11.7 | 39.1 | 4.9 | 15.3 | 107.7 | 66.3 | 28.5 | 61.2 | 72.8 | 8.1 | 7.3 | 21.9 | 3.3 | 22.4 |
| 4 | 30.2 | 48.3 | 34.0 | 8.5 | 16.3 | 30.5 | 23.0 | 31.5 | 16.7 | 28.2 | 34.2 | 27.4 | 19.3 | 71.9 | 9.0 | 8.1 | 18.5 | 3.2 | 16.6 |
| 5 | 22.4 | 20.2 | 14.6 | 16.0 | 14.7 | 38.6 | 22.4 | 19.1 | 15.5 | 22.4 | 22.4 | 11.9 | 16.0 | 122.3 | 10.4 | 8.3 | 22.0 | 1.8 | 43.2 |
| 6 | 21.1 | 16.4 | 8.9 | 12.5 | 16.4 | 27.2 | 15.6 | 24.0 | 16.0 | 15.3 | 13.7 | 15.2 | 12.4 | 68.3 | 6.6 | 13.3 | 26.5 | 2.0 | 28.8 |
| 7 | 8.2 | 10.3 | 6.9 | 9.3 | 10.5 | 17.3 | 27.3 | 13.2 | 25.6 | 19.6 | 9.6 | 27.3 | 10.7 | 68.9 | 3.6 | 3.5 | 12.1 | 2.7 | 13.0 |
| 8 | 7.3 | 12.0 | 11.0 | 5.2 | 9.2 | 10.9 | 18.8 | 15.2 | 10.9 | 9.4 | 13.8 | 8.3 | 11.1 | 49.4 | 3.5 | 4.6 | 11.0 | 0.7 | 12.6 |
| 9 | 6.5 | 5.3 | 4.9 | 2.8 | 6.9 | 19.4 | 6.3 | 8.4 | 8.3 | 3.6 | 8.8 | 2.3 | 6.6 | 33.6 | 2.8 | 3.9 | 4.5 | 0.0 | 6.9 |
| 10 | 3.1 | 2.4 | 2.6 | 1.4 | 8.6 | 7.2 | 3.0 | 1.8 | 3.7 | 1.0 | 3.6 | 0.6 | 4.8 | 24.6 | 1.7 | 1.6 | 2.8 | 0.0 | 5.9 |

| A | M | A | P | R | T | L | L | L |
|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| | L | E | D | F | T | I | E | H |
| | M | F | E | R | | L | | |
| | | N | H | Y | | P | | |
| | | P | K | | | | | |
| | | Q | | | | | | |
| | | R | | | | | | |
| | | T | | | | | | |

**Figure 2.** Pool sequencing of peptides eluted from soluble Qa-1$^b$ refolded with a random nonameric library. Recombinant Qa-1$^b$ heavy chain was refolded in the presence of a random nonameric library and human β2m. Free peptide and β2m were removed by gel filtration, and bound peptide was eluted and analyzed by Edman degradation. The yield of each amino acid in pmol (indicated by single letter code) is shown. Positions where the yield of amino acid is at least 150% of the previous cycle are underlined.

(43) and its importance was confirmed in this study (Table I). The COOH-terminal position is in general a dominant anchor for class I MHC–binding peptides with a high degree of selectivity for one or a few chemically related (usually hydrophobic or positively charged) residues (45, 48).

To further investigate peptide-binding specificity, Qa-1 was folded with a series of peptide pools each containing the Qdm sequence randomized at a single position. Sequencing and amino acid analysis of the initial peptide pools demonstrated similar representation of each amino acid (except cysteine, which was excluded in synthesis) at the randomized positions. Representative reversed-phase HPLC profiles for the pool randomized at P9, before and after folding and elution from Qa-1, are shown in Fig. 3. HPLC fractions were collected and predominant eluted peptides were identified by mass spectrometry and/or Edman sequencing and quantified by integration of HPLC peak area (λ = 210 nm) (Table II). Among 19 peptides in the pool randomized at P2, those with hydrophobic amino acids Met, Ile, or Leu accounted for >80% of the peptides eluted from Qa-1. The most stringent specificity was observed with the pool randomized at P9; a single peptide containing Leu at P9 accounted for 85% of eluted peptide. A variety of amino acids was preferred at each of the other positions tested. Strikingly, the residue found in each position in the natural Qdm sequence was highly enriched among peptides eluted from Qa-1 for each peptide pool that was tested (Table II). These data support the conclusion that Qdm has an ideal or near-ideal primary structure for binding to Qa-1$^b$.

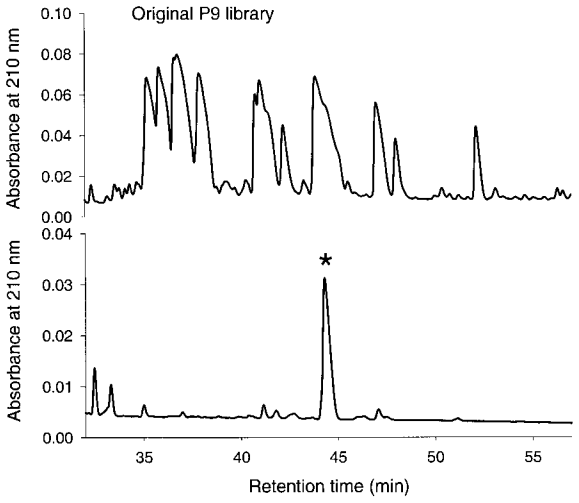


**Figure 3.** Selective incorporation of peptides during in vitro folding of Qa-1$^b$. Folding reactions were carried out in the presence of pools of Qdm peptides each randomized at a single position. After purification, peptide was eluted from the Qa-1 complexes and analyzed by reversed-phase HPLC, mass spectrometry, and Edman sequencing. Shown are HPLC profiles of the Qdm library randomized at P9 and the peptides eluted from Qa-1 that was folded in the presence of this library. *Peak identified as peptide with Leu at P9 (Qdm).

The results from the above experiment with limited peptide pools (Table II) are in reasonable agreement with those obtained using the fully random peptide library (Fig. 2) with respect to amino acids preferred at P2, P5, P6, and P7. Preference for Met and Leu at P2 was identified through both approaches. Similarly, common preferences for Thr at P6 and Pro, Ile, and Leu at P7 were identified. By contrast, few common amino acids were identified as preferred at P3 with the two approaches. It is evident, however, that there is little stringency at this position. The observation that Leu is highly preferred among Qdm peptides randomized at P9 markedly contrasts with the results of our study with the fully random peptide library that showed no enrichment for Leu at this position. This difference might be accounted for by direct or indirect cooperative interactions between amino acid residues at different positions. In the context of the Qdm sequence, the P9 position could be constrained, imposing a stringent requirement for Leu at this position. In the context of other sequences, this preference could conceivably change. Alternatively, the reduced signal attributed to the "washout" that is inevitably associated with later cycles of Edman degradation may have obscured a preference for Leu at P9 in our experiments with the fully random library. Both experimental approaches indicated that Arg and Tyr are preferred at the putative nonanchor P5 position. Thus, side chains at this position can clearly influence association with Qa-1, but whether this side chain is buried (acting as a clas-

**Table II.** *Amino Acid Preference at Specific Positions in Qdm Libraries*

| Position* | Predominant residue | Percentage‡ |
|---|---|---|
| 2 | **Met** | 32 |
| | Ile | 29 |
| | Leu | 20 |
| | Met(O), Glu | 12 |
| | Val | 4.5 |
| | Unassigned | 2.5 |
| 3 | **Ala** | 23 |
| | Ser + Thr | 19 |
| | Ile | 13 |
| | Val | 13 |
| | Leu | 11 |
| | Met | 11 |
| | Gln | 7 |
| | Unassigned | 3 |
| 5 | Ser | 26 |
| | **Arg** + Asn | 21 |
| | Tyr | 12 |
| | His | 10 |
| | Ala | 9.5 |
| | Lys | 7.8 |
| | Thr | 7.3 |
| | Unassigned | 6.4 |
| 6 | Ala | 27 |
| | **Thr** | 26 |
| | Ser | 17 |
| | Val | 7 |
| | His | 6 |
| | Phe | 3.8 |
| | Tyr | 3.7 |
| | Unassigned | 9.5 |
| 7 | Pro | 25 |
| | Met | 22 |
| | **Leu** | 20 |
| | Ile | 20 |
| | Val | 5.1 |
| | Phe | 3.7 |
| | Unassigned | 4.2 |
| 9 | **Leu** | 85 |
| | Val | 2.4 |
| | Met | 1.8 |
| | Phe | 1.7 |
| | Unassigned | 9.1 |

*Qa-1b was folded in the presence of pools of Qdm peptides each randomized at the indicated position.
‡Percentage of peptide eluted from Qa-1b containing the indicated amino acid.

sical anchor) or is solvent exposed (44), influencing binding though surface interactions, remains to be determined.

We performed an additional experiment to explore the possibility that Qa-$1^b$ may have a capacity to bind a diverse repertoire of peptides despite its preference for the Qdm sequence. A peptide library with a complexity of ~500 sequences was synthesized by coupling mixtures of amino acids at P3, P5, P6, and P7 and fixing the other positions with the amino acids found in Qdm. Amino acids included in the mixtures were selected based on preferred residues identified in the experiments described above. Qa-1 was folded in the presence of this library and eluted peptides were analyzed by HPLC; selected peaks were sequenced. A highly diverse set of peptides was observed to associate with Qa-1 under these conditions (data not shown). In addition, four peptides were analyzed from a sequence database search based on the motif identified from the data presented in Table II. One of these four, from influenza nucleoprotein, was observed to bind with relatively high affinity to Qa-$1^b$ (Table I). We conclude that, although Qdm has a near-ideal sequence for binding Qa-1, this class Ib protein has the potential to assemble with a relatively diverse repertoire of peptides.

*The Role of H-2D$^b$ as a Source of Leader Peptide for Inhibition of NK Lysis.* We reported previously that addition of Qdm peptide was required to inhibit lysis of transfected human T2 B cell targets expressing high levels of Qa-$1^b$ in experiments with CD94/NKG2A-positive NK effector cells (23). Endogenous leader peptides were not presented by Qa-1 molecules because T2 cells do not express TAP and leader peptide presentation by Qa-1 requires TAP (11). It is unknown whether these cells express "empty" Qa-1 molecules or Qa-1 associated with some undefined peptides loaded through a TAP-independent mechanism. However, it is clear that Qa-1 molecules expressed in T2 cells were unable to serve as a functional ligands for CD94/NKG2A in the absence of exogenous Qdm (23). The observation that no increase in Qa-1 expression was observed in cells cultured with Qdm suggested that there is peptide dependency in recognition of Qa-1 by the inhibitory receptor (23).

Experiments were performed to further investigate the role of Qdm in generating functional ligand for CD94/NKG2A. In H-$2^b$ cells, the only known source of Qdm is $D^b$ since $K^b$ does not have the correct motif in its leader sequence. The role of $D^b$ in inhibition of NK lysis was investigated in chromium-release assays with targets expressing Qa-1 in the presence or absence of $D^b$. IL-2–activated CD94/NKG2-positive $K^{b-/-}$ NK cells were used as effectors. A small degree of lysis of autologous $K^{b-/-}$ target cells was observed (Fig. 4). This may be due to partial reversal of self-tolerance, which has sometimes been observed when NK cells are cultured in high concentrations of IL-2 (49). However, the $D^b$ molecule on $K^{b-/-}$ targets clearly inhibited lysis because $K^{b-/-}D^{b-/-}$ targets were lysed much better than $K^{b-/-}$ targets. Confirmation that the leader peptide could inhibit NK cells was obtained by incubating $K^{b-/-}D^{b-/-}$ targets with exogenous Qdm; this treatment resulted

in potent inhibition of NK-mediated lysis as shown in a previous study with $K^{b-/-}D^{-/-}$ targets (50). The effect of Qdm appeared to depend on Qa-1 expression since killing of $\beta 2m^{-/-}$ targets, which do not express Qa-1, was not inhibited by exogenous Qdm peptide (Fig. 4). Importantly, killing of $K^{b-/-}D^{b-/-}$ targets was indistinguishable from killing of $\beta 2m^{-/-}$ targets, suggesting that $D^b$ is essential for inhibition of NK cells under our experimental conditions. These results extend previous observations (50, 51) supporting the conclusion that $D^b$ is the only source of peptide that can bind Qa-1 in ConA-activated spleen cells to generate a functional ligand for CD94/NKG2A inhibitory receptors.

*Specificity of CD94/NKG2A Receptors for Qa-1–associated Peptide.* The above experiments and previous studies (23) clearly indicated that Qa-$1^b$–Qdm complexes can serve as ligands for CD94/NKG2A, but the degree to which the receptor is able to discriminate between Qa-1 complexes containing different peptides remained unknown. This question became particularly relevant in light of the observation that Qa-1 has the potential to bind a relatively diverse repertoire of peptides. This issue was further investigated by generating fluorescent soluble tetrameric Qa-$1^b$ complexes bearing Qdm peptides with substitutions at different nonanchor positions. As previously reported (23), tetramers generated from Qa-$1^b$ folded with Qdm peptide bind to 40–50% of splenic NK cells (Fig. 5 A). Staining with tetramers containing the $D^k$ leader sequence, which contains Val instead of Ala at P3, was somewhat reduced compared with Qdm, yet a substantial fraction of NK cells clearly binds this reagent. By contrast, little if any staining was observed with tetramers containing substituted Qdm peptides with Lys at P1, P4, P5, or P8. To confirm the specificity of NK cell staining, a parallel experiment was performed with transfectants expressing high levels of CD94/NKG2A. No tetramer staining was observed with control cells that did not express CD94/NKG2A heterodimer (23). Qa-1 tetramers containing Qdm or the $D^k$ leader peptide stained the transfectants uniformly (Fig. 5 B). No staining was observed with peptides containing substitutions at P4, P5, or P8. The transfectants uniformly reacted with tetramer containing the P1-substituted peptide but the mean fluorescence intensity was substantially reduced, suggesting that this substitution may reduce affinity for CD94/NKG2A. The increased staining of transfected cells compared with splenic NK cells with tetramer containing the P1-substituted peptide probably can be attributed to the greater level of expression of CD94/NKG2A on the transfectants.

Functional experiments were done to confirm the tetramer staining data. As reported previously (23), exogenous

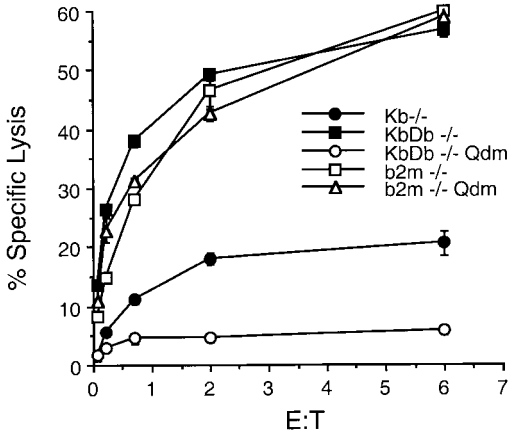


**Figure 4.** $D^b$ is the only cellular source of peptide capable of binding to Qa-$1^b$ and inhibiting NK lysis. IL-2–cultured CD3-NK1.1 plus NKG2 plus splenocytes from $K^{b-/-}$ mice were used as effector cells. Spleen cells from B6 $\beta 2m^{-/-}$, B6 $K^{b-/-}$, and B6 $K^{b-/-}D^{b-/-}$ mice were cultured overnight with ConA (2.5 μg/ml), labeled for 1 h at 37°C with $^{51}Cr$, and used as targets in a standard 4-h $^{51}Cr$-release assay. Qdm peptide (50 μM) was included during culture with ConA, labeling, and lysis phases of the assay for the indicated groups of target cells.

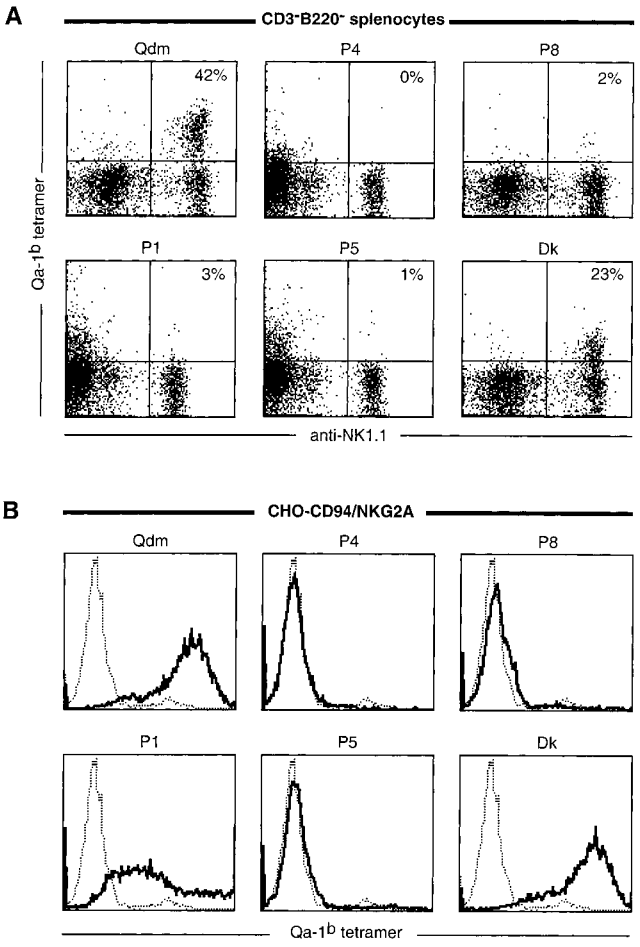


**Figure 5.** Binding of Qa-$1^b$ tetramers containing substituted Qdm peptides to CD94/NKG2A. (A) Flow cytometry profiles were obtained using $CD3^-B220^-$ C57BL/10 splenocytes stained with anti-NK1.1 and Qa-$1^b$ tetramers, prepared with Qdm or substituted Qdm peptides containing Lys at the indicated position. The Dk leader peptide differs from Qdm, containing a Val instead of Ala at P3. Numbers in the upper right hand corner correspond to percentage of $NK1.1^+$ cells that are tetramer positive. (B) Chinese hamster ovary transfectants expressing CD94 and NKG2A were stained with Qa-$1^b$ tetramers (solid line) or streptavidin-allophycocyanin (dotted line).

Qdm peptide is able to markedly inhibit killing of T2 cells expressing Qa-$1^b$ in assays with activated NK cells sorted for expression of CD94/NKG2A using Qa-1–Qdm tetramers (Fig. 6). Qdm does not inhibit lysis mediated by tetramer-negative NK cells that lack expression of CD94/NKG2A. Substantial inhibition was observed with the P1-substituted Qdm variant (Fig. 6). Thus, the P1 substitution had little or no functional consequences despite tetramer staining data suggesting that this substitution may have reduced receptor binding affinity. The $D^k$ leader peptide also inhibited target cell lysis, although the extent of inhibition was somewhat reduced compared with Qdm (Fig. 6). The Val substitution at P3 in the $D^k$ leader is the only known polymorphism in the corresponding leader sequences of murine H-2D and H-2L molecules. Therefore, our results are consistent with the conclusion that functional ligands for CD94/NKG2 can be generated in mice of all haplotypes. No inhibition of killing by tetramer-positive NK cells was observed with substituted Qdm peptides containing Lys at P5 or P8, or with the influenza peptide that had previously been determined to bind Qa-1 with relatively high affinity (Table I).

Together, the tetramer binding data and the functional experiments provide strong evidence that CD94/NKG2A has a high degree of specificity for the primary structure of peptide bound to its Qa-1 ligand. The effect observed with substitutions at P4, P5, or P8 suggests that the receptor may directly contact the side chains of residues in these positions, consistent with a binding interface that may overlap that used for recognition by T cell antigen receptors. This contrasts with the interaction sites recently defined by the crystal structure of the Ly49A NK inhibitory receptor bound to H-$2D^d$ (52). Two sites were identified that are spatially distinct from the T cell receptor binding site, explaining the previous observation that Ly49A recognizes MHC class I–peptide complexes independent of peptide composition (53). There is some evidence, however, that Ly49C and Ly49I may have some degree of peptide selectivity (54–56) The extent to which CD94/NKG2A can discriminate between peptides with chemically conservative substitutions at anchor or solvent-exposed positions remains to be determined. Substitution of smaller nonpolar residues at P4 or P8 with positively charged Lys represents a relatively substantial change in local surface chemistry. In addition, replacement of Pro at P4 may have a significant effect because of the loss of the conformational constraint uniquely associated with Pro. However, a conservative Arg to Lys substitution at P5 also abrogates receptor binding and function. The P5 position may play a particularly important role in receptor recognition.

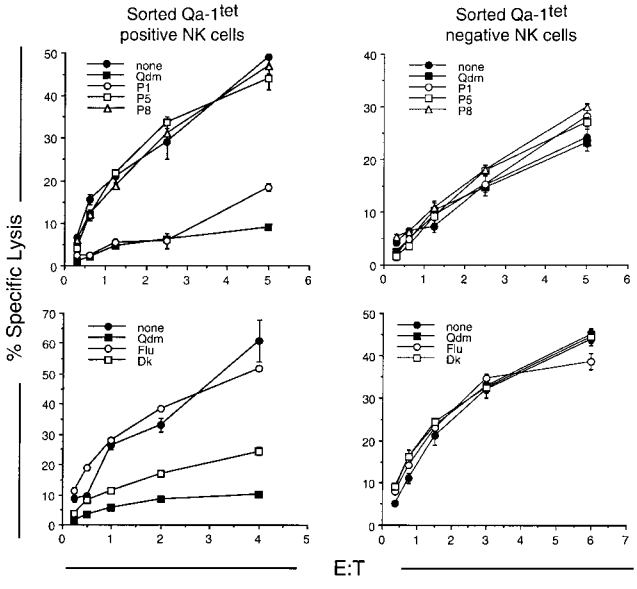


**Figure 6.** Inhibition of NK cell killing by Qa-$1^b$ peptide complexes. IL-2–cultured $CD3^-NK1.1^+$ NK cells were sorted into Qa-$1^b$–Qdm tetramer positive and negative populations. After further culture, the activated NK effector cells were tested for their ability to kill $Cr^{51}$-labeled Qa-$1^b$–transfected T2 cell (T237) targets in the presence of the indicated peptides. Targets were incubated at room temperature overnight with 30 μM peptide and $^{51}Cr$. Peptide (30 μM) was included in the killing assay.

Several studies suggest that sequence variation in HLA-E–bound leader peptides can influence affinity for human CD94/NKG2 receptors. Llano et al. reported that a leader peptide from HLA-Cw7 with Ala instead of Thr at P6 was unable to inhibit NK killing despite good binding to HLA-E as measured by stabilization of HLA-E in TAP-deficient 721.221 cells (35). Polymorphisms in human leader peptide residues P1 (Val, Ile), P7 (Leu, Val), and P8 (Leu, Ile, Val, and Phe) did not affect inhibitory activity, consistent with previous results (27–29, 35). Evidence was also provided suggesting that the HLA-G leader peptide, containing Phe at P8, may be preferentially recognized by the activating receptor CD94/NKG2C compared with other leader peptides (35). Brooks et al. reported that receptor binding was reduced with HLA-E complexes containing the Cw7 leader with Ala at P6 (36). In contrast to the results of Llanno et al. (35), this peptide was observed to inhibit NK killing, but only if included in culture throughout the assay (36). It is possible that HLA-E complexes containing the Cw7 leader peptide may be relatively unstable, explaining the apparent discrepancy. An EBV peptide that had previously been shown to bind HLA-E (57) was unable to form a ligand for soluble CD94/NKG2A, nor did it inhibit killing by NK cells (36). Vales-Gomez et al. demonstrated peptide-dependent differences in the binding kinetics of soluble HLA-E to soluble CD94/NKG2A and CD94/NKG2C (37). For both receptors, the highest affinity interactions were observed with HLA-E containing the leader peptide from HLA-G (Phe at P8). Differences in the binding affinities of a panel of HLA-E complexes containing leader peptides with sequence variation in P2, P3, P6, or P8 further demonstrated specificity for bound peptide (37). Together, the above studies suggest that the human CD94/NKG2 receptors can discriminate between HLA-E complexes containing different leader peptides. The structural basis for discrimination and functional consequences remain to be further characterized.

The specificity of CD94/NKG2A for both Qa-1 and the

primary structure of the Qa-1–bound peptide could make it more difficult for viruses or other microbial pathogens to evade recognition by the immune system. Viral proteins that inhibit components of the class I antigen processing pathway to evade recognition by $CD8^+$ T cells (58) could make an infected cell sensitive to lysis by NK cells if the supply of either Qa-1 or Qdm were also disrupted. Inhibition of TAP activity may not inhibit cell surface expression of the Qa-1 protein (23) but it would certainly inhibit expression of the Qa-1–Qdm complexes (11) required for recognition by inhibitory receptors. To avoid NK recognition, it would be necessary for a virus to provide an alternative peptide capable of binding Qa-1, loaded through a TAP-independent mechanism and mimicking the primary structure of Qdm for recognition by CD94/NKG2A. Just such a mechanism has recently been described by Tomasec et al., who reported that expression of the human cytomegalovirus glycoprotein UL40 can upregulate expression of HLA-E in TAP-deficient cells and protect targets from NK lysis (59). UL40 contains a sequence in its signal peptide exactly identical to a nonameric human HLA-C leader peptide known to bind HLA-E. Murine hepatitis virus E3 glycoprotein contains a sequence identical to Qdm in its leader peptide (60). It is possible that this virus has developed a similar mechanism for escape from NK recognition during coevolution with its murine hosts. Given evidence presented here that Qa-1 has a capacity to bind a relatively diverse repertoire of peptides, it is interesting to speculate that virus-encoded peptides could compete with Qdm for binding to Qa-1 in infected cells without generating an appropriate ligand for CD94/NKG2A inhibitory receptors. This could provide a novel mechanism for NK cell activation.

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