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The Nonclassical MHC Class I Molecule Qa-1 Forms Unstable Peptide Complexes

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The MHC class Ib molecule Qa-1 is the primary ligand for mouse CD94/NKG2A inhibitory receptors expressed on NK cells, in addition to presenting Ags to a subpopulation of T cells. CD94/NKG2A receptors specifically recognize Qa-1 bound to the MHC class Ia leader sequence-derived peptide Qdm. Qdm is the dominant peptide loaded onto Qa-1 under physiological conditions and this peptide has an optimal sequence for binding to Qa-1. Peptide dissociation experiments demonstrated that Qdm dissociates from soluble or cell surface Qa-1b molecules with a $t_{1/2}$ of $\sim 1.5$ h at $37^\circ$C. In comparison, complexes of an optimal peptide (SIINFEKL) bound to the MHC class Ia molecule H-2Kb dissociated with a $t_{1/2}$ in the range from 11 to 31 h. In contrast to Kb, the stability of cell surface Qa-1b molecules was independent of bound peptides, and several observations suggested that empty cell surface Qa-1b molecules might be unusually stable. Consistent with the rapid dissociation rate of Qdm from Qa-1b, cells become susceptible to lysis by CD94/NKG2A+ NK cells under conditions in which new Qa-1b/Qdm complexes cannot be continuously generated at the cell surface. These results support the hypothesis that Qa-1 has been selected as a specialized MHC molecule that is unable to form highly stable peptide complexes. We propose that the CD94/NKG2A-Qa-1/Qdm recognition system has evolved as a rapid sensor of the integrity of the MHC class I biosynthesis and Ag presentation pathway. The Journal of Immunology, 2004, 172: 1661–1669.

The T23-encoded MHC class Ib molecule Qa-1b functions in both innate and adaptive immune responses, regulating NK cell activation and serving as a restriction element for a subpopulation of CD8+ T cells. This murine nonclassical MHC class I molecule assembles with $\beta_2$-microglobulin ($\beta_2$m) and is expressed in a wide range of tissues similar to MHC class Ia molecules. However, Qa-1 is relatively polymorphically expressed at lower levels than MHC class Ia molecules, possibly due to an increased rate of cellular turnover (1). In contrast to classical MHC class I molecules, Qa-1 predominantly binds a single nonameric peptide, Qa-1 determinant modifier (Qdm) (2). Presentation of endogenous Qdm is strictly dependent on TAP, presumably because the Qdm-containing fragment of H-2D/L leader peptides is released into the cytoplasm after cleavage by signal peptide peptidase (3, 5, 6). In vitro folding experiments with peptide libraries demonstrate that Qdm (sequence AMAPRTLLL) has an ideal sequence for binding Qa-1 (7). Nevertheless, Qa-1 can bind other peptides and present them to T cells (8–12). Qa-1 expression is normal in H-2Db and it is only partially reduced in TAP-deficient cells, lacking Qdm and other TAP-dependent peptides (13, 14). The nature of the peptides bound to Qa-1 in TAP−/− cells is unknown.

Qa-1 serves as the dominant and probably exclusive ligand for CD94/NKG2 signaling receptors, which are expressed on a large fraction of NK cells and a subpopulation of CD8+ T cells (14–17). The most highly expressed isoform, CD94/NKG2A, is an inhibitory molecule. Expression of Qa-1/Qdm complexes on target cells inhibits killing by CD94/NKG2A+ NK cells. The CD94/NKG2 MHC class Ib recognition system appears to have an ancient origin in evolution because a homologous system is present in humans. Human CD94/NKG2 receptors recognize the HLA class Ib molecule HLA-E (18–20), which is not a clear homologue of Qa-1 based on overall amino acid sequence comparison. However, these HLA class Ib molecules share an interesting structural feature; they have Ser instead of Thr and Trp at positions 143 and 147. The latter residues are otherwise highly conserved in MHC class I molecules, participating in a canonical hydrogen bond network involving the C terminus of bound peptide (21). Like Qa-1, HLA-E selectively binds MHC class Ia leader sequence-derived peptides with sequences similar to Qdm. Despite considerable sequence differences, Qa-1 and HLA-E have remarkably similar peptide binding specificity. The crystal structure of HLA-E bound to a human leader sequence-derived peptide demonstrated a modified hydrogen bond network and other structural features supporting the conclusion that HLA-E evolved to bind selectively and tightly to a highly homologous set of MHC class Ia-derived leader sequence peptides (22). However, the stability of these peptide complexes has not been directly measured. Recognition of Qa-1 (and HLA-E) by CD94/NKG2A receptors is sensitive to the sequence of Qa-1-bound peptide (7). Thus Qdm dissociation or replacement with other peptides would result in a loss of recognition by CD94/ NKG2A, relieving the inhibitory signal.

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2 Abbreviations used in this paper: $\beta_2$m, $\beta_2$-microglobulin; Qdm, Qa-1 determinant modifier.

3 This work was supported by National Institutes of Health Grants AI33614 and AI05544.

In the present study, we describe experiments that demonstrate that Qa-1\(^{\text{Qdm}}\) peptide complexes are relatively unstable in comparison to peptide complexes generated with an MHC class Ia molecule, H-2K\(^{\text{b}}\), and an optimal binding peptide for this molecule. Evidence is presented supporting the conclusion that empty Qa-1\(^{\text{b}}\) molecules may be unusually stable. Our results support a model in which Qa-1\(^{\text{Qdm}}\) complexes must be continuously replenished on target cells to inhibit recognition by CD94/NKG2A\(^{+}\) NK cells.

**Materials and Methods**

**Mice and reagents**

C57BL/6 (B6) mice, TAP\(^{-/-}\), and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). H-2D\(^{\text{k}}\)-transfected (T2-K\(^{\text{k}}\)) and Qa-1\(^{\text{b}}\)-transfected (T2-37 or J1) cells were incubated with biotin-Qdm-4C, biotin-A3-11 or biotin-SIICFEKL (100 nM) peptide and biotin-Qdm-4C, respectively, in 1 h at room temperature in PBS. The cells were washed extensively in cold PBS and incubated at 37°C in the presence of 200 nM of unlabeled Qdm or SIINFEKL peptide in RPMI 1640 medium (10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin). After the indicated lengths of time, the cells were washed and incubated in fresh medium containing biotin-Qdm-4C (100 nM) for 10 min at room temperature and washed in PBS. Con A blasts were prepared by stimulating 2×10\(^{6}\) spleen cells with Con A (3 \(\mu\)g/ml) for 2-3 days. In some experiments, T2-37 cells or TAP\(^{-/-}\) Con A blasts were incubated for 18 h at 27°C in the presence or absence of Qdm or SIINFEKL to increase surface expression of MHC class I molecules.

**Measuring peptide dissociation rates from recombinant MHC class I molecules**

Purified Qa-1\(^{\text{Qdm}}\), HLA-E/Qdm, and H-2K\(^{\text{b}}\)/SIINFEKL complexes were generated from Escherichia coli-derived proteins as previously described (7, 24). Qa-1\(^{\text{Qdm}}\), HLA-E/Qdm, and H-2K\(^{\text{b}}\)/SIINFEKL complexes were incubated with biotin-Qdm-4C, biotin-A3-11 or biotin-SIINFEKL (1 \(\mu\)M) peptide, respectively, at room temperature to allow for peptide exchange in PBS with 0.01% Nonidet P-40. The next day, 200 \(\mu\)M of unlabeled A3-11, Qdm or SIINFEKL peptide was added to the mixtures and incubated at 37°C. After the indicated lengths of time, the samples were placed on ice to minimize further peptide dissociation. The amount of peptide that remained bound to the MHC class I molecules in each sample was measured by a fluorescence immunoassay modified for measuring MHC class I complexes using an anti-\(\beta_{2m}\) capture Ab (7).

**Measuring peptide dissociation rates from cell lytated-derived MHC class I molecules**

As a cellular source of MHC class I molecules, T2-K\(^{\text{k}}\) (K\(^{\text{k}}\)-transfected T2 cell line) and T2-37 (Qa-1\(^{\text{b}}\)-transfected T2 cell line) cells were incubated with 1 \(\mu\)M biotin-SIINFEKL peptide and biotin-Qdm-4C, respectively, for 1 h at room temperature in PBS. The cells were washed extensively in cold PBS and lysed in lysis buffer (PBS, 1% Nonidet P-40 and 10 mM EDTA) for 1 h at 4°C followed by centrifugation to remove insoluble products. Unlabeled Qdm (200 \(\mu\)M) or SIINFEKL peptide was added to the mixtures and incubated at 37°C. After the indicated lengths of time, the samples were placed on ice to minimize further peptide dissociation. The amount of peptide that remained bound to the MHC class I molecules in each sample was measured by an anti-\(\beta_{2m}\) capture Ab using a europium-based fluorescence immunoassay.

**Flow cytometry**

Cells were plated in 96-well V-bottom plates and were pelleted and resuspended in 50 \(\mu\)l of PBS containing anti-Qa-1\(^{\text{b}}\)-biotin (clone 6A8.6F10.1A6) at a concentration of 0.5 \(\mu\)g/ml. The cells were washed twice and resuspended in PBS containing streptavidin-allophycocyanin (Molecular Probes, Eugene, OR) at 1:1000 and/or anti-K\(^{\text{k}}\)-FITC (clone Y3) at a concentration of 0.5 \(\mu\)g/ml. An isotype control Ab conjugated with the respective fluorescent or biotinylation tag was used for negative control staining of each specific Ab. After 30 min on ice, the cells were washed with PBS and the fluorescence intensity was measured on a FACScan flow cytometer (BD Biosciences). Finally, the data were analyzed using CellQuest computer software (BD Biosciences).

**Measuring peptide dissociation rates from cell surface MHC class I molecules**

H-2K\(^{\text{k}}\)-transfected (T2-K\(^{\text{k}}\)) and Qa-1\(^{\text{b}}\)-transfected (T2-37 or J1) cells were incubated with 1 \(\mu\)M of biotin-SIIFCEKL peptide and biotin-Qdm-4C, respectively, in 1 h at room temperature in PBS. The cells were washed extensively in cold PBS and incubated at 37°C in the presence of 200 \(\mu\)M of unlabeled Qdm or SIINFEKL peptide in RPMI 1640 medium (10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin). After the indicated lengths of time, the cells were washed and placed on ice to minimize further peptide dissociation. The cells were washed and incubated with streptavidin-allophycocyanin (Molecular Probes) for 30 min on ice. The amount of biotin-peptide that remained bound to the cell surface was measured by flow cytometry. In some experiments, the cells were lysed in lysis buffer after incubations at 37°C and the amount of biotin-peptide that remained bound to the MHC class I molecules was measured by an anti-\(\beta_{2m}\) capture Ab using a europium-based fluorescence immunoassay.

**Measuring the decay rates of cell surface MHC class I molecules**

T2-37 cells, T2-K\(^{\text{k}}\) cells or Con A blasts derived from B6, H-2D\(^{\text{d}}\)-transfected and TAP\(^{-/-}\) mice were placed in RPMI 1640 medium with brefeldin A (20 \(\mu\)M). After timed incubations at 37°C, the cells were removed and placed on ice. The expression of Qa-1\(^{\text{b}}\) and H-2K\(^{\text{k}}\) was measured using flow cytometry. In some experiments, the T2-37 cells and T2-K\(^{\text{k}}\) cells were pre-labeled with Qdm or SIINFEKL, respectively, in PBS for 1 h at room temperature and washed in PBS. Con A blasts were prepared by stimulating 2×10\(^{6}\) spleen cells with Con A (3 \(\mu\)g/ml) for 2-3 days. In some experiments, T2-37 cells or TAP\(^{-/-}\) Con A blasts were incubated for 18 h at 27°C in the presence or absence of Qdm or SIINFEKL to increase surface expression of MHC class I molecules.

**Measuring peptide-receptive Qa-1\(^{\text{b}}\) molecules**

T2-37 cells were placed in RPMI 1640 medium with brefeldin A (20 \(\mu\)M). After timed incubations at 37°C, the cells were removed and placed in medium containing biotin-Qdm-4C (10 \(\mu\)M) for 10 min at room temperature. The cells were placed immediately on ice following the 10 min incubation to prevent further decay of Qa-1\(^{\text{b}}\) molecules. The expression of peptide-receptive Qa-1\(^{\text{b}}\) was quantified by measuring the amount of biotin-peptide that was bound to the cell by flow cytometry.

**Calculation of half-life of MHC class I molecules and MHC-peptide complexes**

Assuming first order kinetics, \(\log (B/B_0)\) was plotted against time and a linear regression analysis was performed to determine the rate constant (slope of the line). The half life of MHC class I molecules and MHC-peptide complexes was calculated by dividing \(\log_2\) by the rate constant. The value of B was obtained by subtracting background fluorescence from the europium (obtained by immunoassay), allophycocyanin or FITC (obtained by flow cytometry) fluorescence of each sample. \(B_0\) is the B value obtained at \(t = 0\).

**Preparation of dendritic cells**

The bone marrow of the tibia and femur of B6 or H-2D\(^{\text{d}}\)-transfected mice were flushed with a syringe using cold RPMI 1640 medium. Single cell suspensions were obtained and cultured in DMEM (15% FBS, 100 U/ml penicillin, 100 U/ml streptomycin) containing GM-CSF (10 ng/ml) and IL-4 (100 ng/ml) (PeproTech, Rocky Hill, NJ) in 12-well plates. After 6 days of culture, CD11c\(^{+}\) dendritic cells were purified using the MACS separation system (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s guidelines. Briefly, the cultured bone marrow cells were washed twice with MACS buffer (PBS without Ca\(^{++}\) supplemented with 0.5% BSA and 2 mM EDTA) and resuspended in MACS buffer containing the appropriate amount of CD11c magnetic beads. After a 30 min incubation at 4°C, CD11c\(^{+}\) cells were purified from the cell suspension using an LS\(^{+}\) column, washed once and resuspended in DMEM containing LP5 from Salmonella enteriditis (100 ng/ml). The cells were incubated overnight at 37°C and used as targets for NK cell-mediated lysis.
Preparation of NK cells

Single cell suspensions of erythrocyte-depleted spleen cells were incubated with anti-NKG2A/Ce-biotin Ab (BD PharMingen) in PBS for 30 min at 4°C. The cells were washed and incubated in PBS with magnetic beads conjugated to streptavidin. The cells were washed and NKG2A+/NK cells were purified using the MACS separation system (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s guidelines. The purified cells were cultured in lymphokine-activated killer medium (α-MEM supplemented with 10 mM HEPES, 2 × 10−8 M 2-μg/ml FCS, 100 μM penicillin, 100 μM streptomycin) (Life Technologies, Rockville, MD) supplemented with murine IL-2 (1000 U/ml) (PeproTech) for 6 days and FACSVantage-sorted into purified NKG2A+ NK cells on the day of the cytotoxicity assay.

Cytotoxicity assays

T2–37 cells, H-2Dβ−/− dendritic cells and H-2Dβ+/− Con A blasts were labeled with 51Cr in the presence or absence of Qdm (50 μM) for 1–2 h at room temperature. The cells were washed extensively in cold RPMI 1640 medium and plated in 96-well V-bottom plates at 5000 cells/well at 37°C. Some cells were incubated with Qdm (50 μM) during the assay as indicated. One hour later, NKG2A+/NK cells were added to the wells. In some experiments, dendritic cells from B6 mice were labeled with 51Cr in the presence or absence of Qdm (100 μM) or emetine (100 μM) for 5 h at 37°C. The cells were washed extensively in cold RPMI 1640 medium and plated in 96-well V-bottom plates at 5000 cells/well at 37°C and NKG2A+/NK cells or NKG2A−NK cells were added immediately to the wells. The cells that were pretreated with Qdm (100 μM) also received the same concentration of Qdm during the assay. Four hours later, 51Cr release in the supernatant was detected using a gamma counter and the percentage of specific lysis was calculated using the formula: specific lysis (%) = (sample release − spontaneous release)/total release × spontaneous release × 100, in which spontaneous release is 51Cr release in supernatants of wells without NK cells and total release is 51Cr content of target cells lysed in 1% Triton X-100.

Some cells were incubated with Qdm (50 μM) during the assay as indicated. One hour later, NKG2A+/NK cells were added to the wells. In some experiments, dendritic cells from B6 mice were labeled with 51Cr in the presence or absence of Qdm (100 μM) or emetine (100 μM) for 5 h at 37°C. The cells were washed extensively in cold RPMI 1640 medium and plated in 96-well V-bottom plates at 5000 cells/well at 37°C and NKG2A+/NK cells or NKG2A−NK cells were added immediately to the wells. The cells that were pretreated with Qdm (100 μM) also received the same concentration of Qdm during the assay. Four hours later, 51Cr release in the supernatant was detected using a gamma counter and the percentage of specific lysis was calculated using the formula: specific lysis (%) = (sample release − spontaneous release)/total release × spontaneous release × 100, in which spontaneous release is 51Cr release in supernatants of wells without NK cells and total release is 51Cr content of target cells lysed in 1% Triton X-100.

Results

Qdm dissociates rapidly from recombinant Qa-1β molecules

To investigate the stability of Qa-1β/Qdm complexes, the dissociation rate of Qdm from recombinant Qa-1β molecules was compared with the dissociation rate of SIINFEKL peptide from recombinant H-2Kβ molecules. H-2Kβ-SIINFEKL was chosen as a representative classical MHC class I molecule-peptide complex, because both Qdm and SIINFEKL peptides possess ideal sequences for binding to Qa-1β and H-2Kβ, respectively (7, 25–29). Peptide binding was detected by using biotinylated analogues of Qdm and SIINFEKL that have a cysteine substitution at position 4, to introduce a thiol group that can be readily biotinylated. This amino acid substitution does not alter the binding properties of Qdm or SIINFEKL to their respective MHC class I molecules (7 and data not shown). An anti-β2m mAb capture europium fluorescence immunoassay was used to detect the fraction of recombinant Qa-1β molecules that remained bound to biotinylated Qdm after timed and temperature-controlled incubations in the presence of excess unlabelled Qdm peptide. In agreement with a previous report (30) Qdm was found to form a stable complex with Qa-1β at 4°C. Peptide dissociation was minimal over a 6 h time course, with a t1/2 of 50 h (Fig. 1A). In contrast to the long half-life observed at 4°C, Qdm was observed to dissociate rapidly from Qa-1β molecules at 37°C, with a t1/2 of 93 ± 8 min (n = 11) (Fig. 1A).

In comparison, the dissociation of SIINFEKL from recombinant H-2Kβ molecules was slow at 37°C, with a t1/2 of 1951 ± 527 min (n = 7) (Fig. 1A).

We next tested whether the dissociation rate of a peptide from HLA-E, the human functional ortholog of Qa-1β, was also fast. Instead of Qdm, a peptide derived from the human HLA-A2 leader sequence (A3-11) was used. Interestingly, the half-life of HLA-E/
A3–11 peptide complexes was found to be 13 ± 1.2 min (n = 3) (Fig. 1A). This was ∼8-fold shorter than Qa-1\(^{\text{b}}\)/Qdm and 100–200-fold shorter than H-2K\(^{\text{b}}\)/SIINFEKL. Taken together, these data suggest that both Qa-1\(^{\text{b}}\) and HLA-E form peptide complexes that are significantly less stable than classical MHC class I molecules at physiological temperature, even when bound to peptide with an ideal binding sequence.

Qdm dissociates rapidly from Qa-1\(^{\text{b}}\) derived from Qa-1\(^{\text{b}}\)-transfected cell lines

To exclude the possibility that the short half-life of Qa-1\(^{\text{b}}\)/Qdm complexes was due to an artifact of recombinant proteins that lack glycosylation, the dissociation of Qdm from Qa-1\(^{\text{b}}\) molecules derived from a Qa-1\(^{\text{b}}\)-transfected mammalian cell line, T2–37, was measured. As shown in Fig. 1B, the half-time of Qa-1\(^{\text{b}}\)/Qdm complexes derived from detergent-solubilized T2–37 was 71 ± 4.1 min (n = 3) at 37°C. This value was similar to the half-life observed with the recombinant Qa-1\(^{\text{b}}\)/Qdm complexes. In comparison, the \(t_{1/2}\) of H-2K\(^{\text{b}}\)/SIINFEKL derived from an H-2K\(^{\text{b}}\)-transfected cell line, T2-K\(^{\text{b}}\), was 662 ± 175 min (n = 3). These data suggest that soluble Qa-1\(^{\text{b}}\)/Qdm complexes are significantly less stable than soluble H-2K\(^{\text{b}}\)/SIINFEKL complexes regardless of their source.

Next, the stability of membrane-bound Qa-1\(^{\text{b}}\)/Qdm complexes was examined. A Qa-1\(^{\text{b}}\)-transfected cell line, J1, was preloaded with biotinylated Qdm, washed, and incubated at 37°C in the presence of excess unlabeled Qdm. The amount of Qa-1\(^{\text{b}}\) molecules that remained bound to biotinylated Qdm was determined by fluorescence immunoassay after the cells were solubilized in detergent. Similar to soluble complexes, the \(t_{1/2}\) of membrane-bound Qa-1\(^{\text{b}}\)/Qdm was 85 ± 9.2 min (n = 4) (Fig. 1C). The \(t_{1/2}\) of membrane-bound Qa-1\(^{\text{b}}\)/Qdm and H-2K\(^{\text{b}}\)/SIINFEKL was also compared using an alternative approach. Instead of an immunooassay, the quantity of biotinylated peptide that remained bound to the surface of the cells was measured by flow cytometry. Using this approach, the \(t_{1/2}\) of Qa-1\(^{\text{b}}\)/Qdm and H-2K\(^{\text{b}}\)/SIINFEKL complexes were found to be 54 ± 6.2 min (n = 5) and 682 ± 97 min (n = 4), respectively (Fig. 1D). In all of the previously discussed experiments, peptide was labeled through a cysteine at another nonanchor position, P8 (AMAPTRLCL). Substitutions in the nonanchor P4 position do not affect Qdm binding affinity as measured in competition binding assays (7). However, it was possible that this substitution might reduce the stability of peptide complexes as measured by peptide dissociation kinetics. We therefore measured the rate of dissociation of another Qdm variant (Qdm-SC) that was labeled through a cysteine at another nonanchor position, P8 (AMAPTRCL), and similar results were obtained (data not shown). Taken together, these data indicate that both soluble and membrane-bound forms of Qa-1\(^{\text{b}}\)/Qdm complexes are unstable compared with H-2K\(^{\text{b}}\)/SIINFEKL complexes.

The impact of rapid peptide dissociation on Qa-1\(^{\text{b}}\) cell surface expression

To see whether the kinetics of Qdm peptide dissociation from Qa-1\(^{\text{b}}\) molecules correlated with disappearance of Qa-1\(^{\text{b}}\) expression from the cell surface, the \(t_{1/2}\) of Qa-1\(^{\text{b}}\)/Qdm complexes was compared with the \(t_{1/2}\) of Qa-1\(^{\text{b}}\) surface expression by flow cytometry. Because T2–37 cells are TAP-deficient and thereby lack an endogenous source of Qdm, T2–37 cells were preincubated with Qdm to allow Qa-1\(^{\text{b}}\)/Qdm complexes to preform on the cell surface. The expression of Qa-1\(^{\text{b}}\) on these cells was detected by an anti-Qa-1\(^{\text{b}}\) mAb (6A8.6F10) after timed incubations at 37°C in the presence of brefeldin A to prevent delivery of newly synthesized molecules to the cell surface. Surprisingly, the \(t_{1/2}\) of Qa-1\(^{\text{b}}\) surface expression was 2–3-fold longer than the \(t_{1/2}\) of the Qa-1\(^{\text{b}}\)/Qdm complexes (Fig. 2A). Interestingly, loading with exogenous Qdm did not significantly prolong the \(t_{1/2}\) of Qa-1\(^{\text{b}}\) molecules on T2–37 cells (Fig. 2B). Qa-1\(^{\text{b}}\) molecules were relatively stable under serum-free conditions, ruling out the possibility that exogenous peptides in the serum substantially stabilized the Qa-1\(^{\text{b}}\) molecules (Fig. 2B). Similar results were obtained in experiments using protein synthesis inhibitors instead of brefeldin A to block delivery of newly synthesized Qa-1 to the cell surface (data not shown). Experiments were performed with Con A blasts from B6, D\(^{b/-/-}\), and TAP\(^{-/-}\) mice to further investigate the role of peptides in stabilizing cell surface Qa-1\(^{\text{b}}\) molecules. Qa-1\(^{\text{b}}\) molecules on wild-type B6 cells should be predominantly occupied by Qdm. In the absence of a source of Qdm from the D\(^{b}\) leader sequence, D\(^{b/-/-}\) mice may be predominantly occupied by a heat shock protein 60 peptide (31). In TAP-deficient cells, the Qa-1\(^{\text{b}}\) molecules are presumably empty. As shown in Fig. 2C, the half-lives of surface Qa-1\(^{\text{b}}\) were similar, ranging from 155 to 172 min for the three cell types. As with T2–37 cells, loading with Qdm peptide had little or no effect in prolonging surface half-life on TAP\(^{-/-}\) lymphoblasts (Fig. 2D).

In many previous studies it has been demonstrated that optimal binding peptides markedly stabilize MHC class Ia molecules on the surface of TAP-deficient cells. Consistent with these previous observations, the half-life of K\(^{\text{b}}\) molecules on T2-K\(^{\text{b}}\) and TAP\(^{-/-}\) lymphoblasts was markedly increased after loading with the K\(^{\text{b}}\)–binding peptide SIINFEKL (Fig. 2, E and F). In the absence of peptide, K\(^{\text{b}}\) molecules decayed rapidly, with \(t_{1/2}\) of ∼10 min. After peptide loading, the \(t_{1/2}\) increased to 6 h or more, depending on the cell type.

The possibility that peptide has a relatively small impact in stabilizing Qa-1\(^{\text{b}}\) molecules was further supported by analysis of the Qa-1\(^{\text{b}}\) expression on TAP\(^{-/-}\) lymphoblasts. In contrast to K\(^{\text{b}}\), Qa-1\(^{\text{b}}\) expression is only slightly reduced in TAP-deficient cells as compared with wild-type B6 cells (Fig. 3, A and B), consistent with previous studies (13, 14). D\(^{b/-/-}\) cells, lacking Qdm, express wild-type levels of Qa-1\(^{\text{b}}\) (Fig. 3A) (13, 23). Overnight incubation at 27°C leads to a moderate increase in Qa-1\(^{\text{b}}\) expression on TAP\(^{-/-}\) lymphoblasts (Fig. 3A), indicating the Qa-1\(^{\text{b}}\) molecules in these cells have increased stability at lower temperature. However, Qdm peptide did not lead to a further increase in surface expression. This result contrasts with the substantial increase in K\(^{\text{b}}\) expression observed when SIINFEKL was included in the culture (Fig. 3B). Together, these findings suggest that peptide occupancy may have a relatively small effect on the stability of Qa-1\(^{\text{b}}\) molecules.

An important caveat in interpreting the previously discussed experiments relates to the specificity of the mAb (6A8.6F10.1A6) used to measure surface Qa-1\(^{\text{b}}\) molecules. The mAb available for measuring Qa-1\(^{\text{b}}\) was generated to a synthetic peptide from a C-terminal segment of the ε2 domain, and this mAb recognizes both native and denatured Qa-1\(^{\text{b}}\). Thus, the measured Qa-1\(^{\text{b}}\) expression includes both native molecules and any denatured molecules that remain at the cell surface. As a surrogate measure of native Qa-1\(^{\text{b}}\), we determined the decay of peptide-receptive molecules. In kinetic experiments at room temperature, the binding of biotin-labeled Qdm to cell surface Qa-1\(^{\text{b}}\) molecules on T2–37 cells was rapid, reaching ∼90% saturation in 10 min (Fig. 5A). This reflects rapid peptide binding by the presumably empty Qa-1\(^{\text{b}}\) molecules expressed on the surface of these TAP-deficient cells. T2–37 cells were incubated at 37°C in the presence of brefeldin A, and peptide-receptive Qa-1\(^{\text{b}}\) molecules were measured at various time points by incubating samples with biotin-labeled Qdm for 10 min at room temperature, followed by staining with streptavidin-allophycocyanin at 4°C. Peptide receptive Qa-1\(^{\text{b}}\) molecules were observed to
The continuous presence of Qdm is required for efficient inhibition of NKG2A+ NK cell-mediated lysis

The significance of the Qa-1\(^b/Qdm\) complex lies within its specific ability to bind the CD94/NKG2A inhibitory receptor expressed on a subset of NK cells. The CD94/NKG2A receptor does not recognize Qa-1\(^b\) molecules that are bound to peptides other than Qdm. Because Qa-1\(^b/Qdm\) complexes are relatively unstable compared with optimal classical MHC class I-peptide complexes, we hypothesized that a continuous source of Qdm may be required to maintain sufficient levels of Qa-1\(^b/Qdm\) complexes on the cell surface for efficient inhibition of CD94/NKG2A+ NK cell-mediated lysis. To test this hypothesis, T2–37 cells were incubated in the presence or absence of exogenous Qdm peptide. These cells were washed extensively and added to IL-2-activated NKG2A+ NK cells. NKG2A+ NK cell-mediated cytotoxicity was significantly inhibited when T2–37 cells were given exogenous Qdm before and during the cytotoxicity assay. However, only partial inhibition was observed when

FIGURE 2. The expression of Qa-1\(^b\) is stable compared with Qa-1\(^b/Qdm\) complexes and is not further stabilized by adding exogenous Qdm. A, T2–37 cells were loaded with Qdm (1 \(\mu M\)) or biotinylated Qdm (1 \(\mu M\)) for 1 h in PBS at room temperature, washed, and incubated at 37°C with brefeldin A (10 \(\mu M\)) in serum-containing medium. The dissociation of Qdm (\(\square\)) and the decay of Qa-1\(^b\) expression (\(\bigcirc\)) were measured by flow cytometry. B, The decay of Qa-1\(^b\) expression on T2–37 cells incubated at 37°C with brefeldin A in serum-containing (\(\square\)) or serum-free medium (\(\bigcirc\)) in the absence of Qdm was measured by flow cytometry. C, The decay of H-2K\(^b\) expression on Con A blasts from B6 mice (\(\square\)) and Qa-1\(^b\) expression on Con A blasts derived from B6, H-2D\(^b\)\(^+\)\(^/-\) (\(\bigcirc\)), and TAP\(^+\)\(^/-\) (\(\triangle\)) mice incubated at 37°C with brefeldin A was measured by flow cytometry. D, The decay of Qa-1\(^b\) expression at 37°C in the presence of brefeldin A was measured on Con A blasts from TAP\(^+\) mice that were previously incubated for 18 h at 27°C (\(\square\)) or 27°C + Qdm (100 \(\mu M\)) (\(\bigcirc\)). E, T2-K\(^b\) cells were labeled with SIINFEKL (1 \(\mu M\)) or biotinylated SIINFEKL (1 \(\mu M\)) for 1 h in PBS at room temperature, washed, and incubated at 37°C with brefeldin A in serum-containing medium. The dissociation of SIINFEKL (\(\square\)) and the decay of H-2K\(^b\) expression (\(\bigcirc\)) were measured by flow cytometry. The decay of H-2K\(^b\) expression was also measured on T2-K\(^b\) cells that were not preloaded with SIINFEKL (\(\bigcirc\)). F, The decay of H-2K\(^b\) expression at 37°C in the presence of brefeldin A was measured on Con A blasts from TAP\(^+\) mice that were previously incubated for 18 h at 27°C (\(\square\)) or 27°C + SIINFEKL (1 \(\mu M\)) (\(\bigcirc\)).

FIGURE 3. The expression of Qa-1\(^b\) is only slightly diminished on TAP\(^+\) Con A lymphoblasts compared with wild-type Con A lymphoblasts. A, The expression level of Qa-1\(^b\) and H-2K\(^b\) (\(\square\)) was measured on Con A lymphoblasts derived from B6 (left panels), H-2D\(^b\)\(^+\)\(^/-\) (center panels), and TAP\(^+\) mice (right panels) by flow cytometry. The number in each histogram plot represents mean fluorescence intensity. Staining with isotype control Ab is shown as a thin line in each histogram plot.
Studies analyzing peptides eluted from purified Qdm complexes demonstrated very few peptides, with Qdm being the only sequence that could be specifically identified (2, 4). Furthermore, experiments analyzing the in vitro folding of recombinant QA-1b H chain and β2m in the presence of peptide libraries demonstrated strong preference for the amino acids found at corresponding anchor positions in Qdm (7). Thus Qdm has an ideal sequence for the amino acids found at corresponding anchor positions in Qdm (7). Thus Qdm has an ideal sequence for anchoring interactions and is unlikely that any other peptide has a significantly higher affinity for QA-1b.

Kurepa et al. (31) in a previous study analyzed the kinetics of dissociation of radiolabeled Qdm peptide from cell surface QA-1b molecules, observing a t1/2 of ~10 h at 4°C. However, the stability of HA/Qdm was not measured at physiological temperature. We also observed a long t1/2 (~50 h) at 4°C when measuring the dissociation of biotinylated Qdm from soluble or cell surface QA-1b molecules. Thus, these peptide complexes are quite stable at 4°C. However, QA-1b/Qdm peptide complexes were much less stable at 37°C, with t1/2 ranging from 46 to 100 min. The observed peptide dissociation rates were similar with unglycosylated soluble recombinant QA-1b, detergent-solubilized QA-1b, and cell surface QA-1b molecules. Similar results were obtained with Qdm peptide variants labeled through two different nonanchor positions in the peptide. Thus the QA-1b/Qdm complex has a relatively short half-life at physiological temperature. By contrast, the K0/SINFEKL complex was observed to have a t1/2 at 37°C ranging from 11 to 31 h, depending on the experimental conditions, consistent with previous reports (28, 29, 32). Although data in the literature are relatively scanty, it appears that the stability of K0/SINFEKL complexes is generally representative of MHC class Iα complexes.
bearing peptides with optimal binding motifs (29, 33–36). Given evidence that Qdm has an ideal sequence for binding Qa-1b, we conclude that Qa-1 differs from MHC class Ia molecules in that it cannot form peptide complexes that are long-lived at physiological temperature.

HLA-E is the functional ortholog of Qa-1 in humans. Although HLA-E and Qa-1 are not clear homologues based on amino acid sequence, they serve as ligands for homologous CD94/NKG2 receptors on NK cells and they selectively bind highly homologous MHC class Ia leader sequence-derived peptides (14, 18–20). In a study analyzing the peptide-binding motif of HLA-E, based on in vitro folding with peptide libraries, we observed that the peptide binding specificity of HLA-E is remarkably similar to that of Qa-1b. In the present study, we observed that a biotin labeled form of the HLA-A2 leader sequence-derived peptide, VMACRTLVL (A3-11), dissociates from recombinant HLA-E at 37°C with a t1/2 of 11 min. Similar results were obtained in several types of assays with the recombinant protein (data not shown). Clearly this needs further investigation with glycosylated and cell surface HLA-E molecules. Nevertheless, these results suggest that HLA-E peptide complexes, like Qa-1, are relatively unstable at physiological temperature. HLA-E and Qa-1 share a structural feature not found in other MHC class I molecules. In other MHC class I molecules, the conserved residues Thr at position 143 and Trp at position 147 participate in a canonical hydrogen bond network with main chain atoms in the C terminus of bound peptide. This hydrogen bond network has been shown to play a major role in stabilizing MHC class I-peptide complexes (21). In HLA-E and Qa-1, the amino acids at positions 143 and 147 are replaced with Ser. The crystal structure of HLA-E bound to a leader sequence-derived peptide demonstrated an alternative hydrogen bond network, and the overall structure supported the conclusion that HLA-E is adapted to bind tightly and selectively to a highly homologous set of peptides derived from MHC class Ia leader sequences (22). Our results, however, suggest that these complexes are relatively unstable compared with many MHC class Ia-peptide complexes. Based on this, we hypothesize that the amino acid replacements in HLA-E and Qa-1 that prevent the formation of the canonical hydrogen bond network reduce the basal energy of stabilization of HLA-E and Qa-1 peptide complexes, thus reducing the maximal stability that can be achieved in complexes containing peptides with ideal anchor residues. Extending this hypothesis, we speculate that the reduced diversity of peptides bound to these molecules under physiological loading conditions in vivo reflects a requirement for near ideal peptide anchors to form complexes stable enough to reach the cell surface. Thus, unique structural features of these molecules that reduce the basal stability of peptide complexes may, as a consequence, enhance the selectivity of these molecules for presenting a narrow repertoire of MHC class Ia leader sequence-derived peptides with ideal anchor residues.

In experiments using a mAb with specificity for both native and denatured Qa-1b molecules, the half-life of cell surface Qa-1b

FIGURE 6. The continuous presence of Qdm is required for efficient inhibition of NKG2A+ NK cell-mediated lysis. A, T2–37 cells, Con A blasts from H-2D b−/− mice (B), or CD11c−sorted bone marrow-derived dendritic cells from H-2D b−/− mice (C) were labeled with 51Cr for 2 h at room temperature in the presence or absence of Qdm (50 μM). The cells were washed extensively and plated in a 96-well plate in the presence or absence of Qdm as indicated. After 1 h at 37°C, purified IL-2-activated NKG2A+ NK cells were added to the targets and 51Cr release in the supernatant was detected 4 h later. The results are expressed as mean the percentage of specific lysis ± SD of triplicate wells. The results are representative of at least three independent experiments.

FIGURE 7. Emetine-treated dendritic cells are susceptible to NKG2A+ but not NKG2A- NK cell-mediated lysis. A, CD11c−sorted bone marrow-derived dendritic cells from B6 mice were labeled with 51Cr for 5 h at 37°C without any treatment (○), in the presence of Qdm (100 μM) (●), with emetine (100 μM) (▲), or with emetine and Qdm (▲). The cells were washed extensively and plated in a 96-well plate with purified IL-2-activated NKG2A+ or NKG2A- NK cells (B). The cells that were preincubated with Qdm peptide were also given the same concentration of Qdm peptide during the cytotoxicity assay. The results are expressed as mean the percentage of specific lysis ± SD of triplicate wells. The results are representative of at least three independent experiments.
(~180 min) was observed to be significantly longer than the half-life of Qa-1\textsuperscript{b}/Qdm peptide complexes (46–100 min). This probably reflects a delay in the removal of Qa-1\textsuperscript{b} molecules after Qdm dissociation and denaturation. It will be important to analyze the half-life of surface Qa-1\textsuperscript{b} with a conformation specific mAb, when this reagent becomes available. However, experiments measuring the loss of peptide-receptive Qa-1\textsuperscript{b} molecules on the surface of TAP-deficient cells suggest that peptide-free Qa-1 molecules have a t\textsubscript{1/2} of 39–60 min. By contrast, the K\textsuperscript{b} molecules on the surface of TAP-deficient cells were observed to decay with a t\textsubscript{1/2} of ~10 min in experiments with a conformation-dependent mAb (Y3). In addition, the turnover of surface Qa-1\textsuperscript{b} (native plus denatured) on TAP\textsuperscript{−/−} cells was at best minimally reduced by Qdm peptide loading. By contrast, K\textsuperscript{b} molecules on TAP\textsuperscript{−/−} cells were markedly stabilized by loading with SIINFEKL peptide, as reported in many previous studies. The possibility that empty Qa-1\textsuperscript{b} molecules are unusually stable needs further investigation. Qa-1\textsuperscript{b} can use a TAP-independent endosomal processing pathway to present an insulin peptide to Qa-1\textsuperscript{b}-restricted T cells (8, 9). The relative stability of empty Qa-1\textsuperscript{b} molecules and the rapid dissociation of peptide from this protein may make Qa-1\textsuperscript{b} particularly well suited to participate in peptide exchange reactions at the cell surface or during recycling through early endosomes. Whereas conventional MHC class I molecules would be expected to rapidly denature after peptide dissociation, Qa-1\textsuperscript{b} may be stable for relatively long time periods, providing an opportunity to bind and present new peptides during recycling through early endosomes. The resulting complexes, however, are likely to be short-lived, requiring a continuous source of Ag to be maintained at the cell surface. Thus Qa-1\textsuperscript{b} might be different from other MHC class I molecules in its capacity to present exogenous Ag through an endosomal pathway similar to MHC class II molecules.

HLA-E, in contrast to Qa-1\textsuperscript{b}, is highly dependent on leader sequence-derived peptides for expression and stabilization. Little or no HLA-E is expressed on TAP-deficient cells or cells lacking appropriate MHC class Ia leader peptides (37). We would predict that the decay of cell surface HLA-E molecules would be rapid under conditions in which delivery of new molecules to the cell surface is blocked. HLA-E would not be expected to efficiently present exogenous Ags through a peptide exchange mechanism.

CD94/NKG2A NK cell inhibitory receptors specifically recognize Qa-1\textsuperscript{b}/Qdm complexes and single amino acid substitutions in the Qdm peptide sequence prevent recognition (7). Thus, inhibition of NK cell activation and cytotoxicity is regulated not by the total expression of Qa-1\textsuperscript{b} on target cells, but rather by the expression of specific peptide complexes. Based on a t\textsubscript{1/2} for Qa-1\textsuperscript{b}/Qdm complexes of ~1–1.5 h, one would expect a relatively rapid loss of inhibitory ligands under conditions in which new Qa-1\textsuperscript{b}/Qdm complexes cannot be delivered to the cell surface. The results of peptide washout experiments presented in this study are consistent with this idea. NK cytotoxicity assays were performed with Qa-1\textsuperscript{b}-expressing target cells lacking an endogenous source of Qdm. Cytotoxicity was markedly inhibited under conditions where the Qa-1\textsuperscript{b} molecules on these cells were preloaded with exogenous Qdm peptide and Qdm was included in the 4-h chromium release assay. However, killing activity was partially restored when Qdm-loaded cells were washed and incubated with NK cells in the absence of additional Qdm peptide. In similar experiments, Gays et al. (38) observed little or no reversal of inhibitory activity in peptide washout experiments, concluding that the inhibitory Qa-1\textsuperscript{b}/Qdm peptide complexes are stable for at least 4 h at 37°C. The difference in our results may be due to differences in experimental conditions. The extent of functional inhibition is likely to depend on a variety of parameters, including the population of NK cells used in the assay, the level of expression of CD94/NKG2A on the effecter cells, and the expression of NK activating receptors and their ligands, in addition to the density of Qa-1\textsuperscript{b}/Qdm on the target cells. In the present study, experiments with the irreversible protein synthesis inhibition inhibitor emetine provided further support for the idea that a continuous supply of newly synthesized Qa-1\textsuperscript{b}/Qdm complexes is required to inhibit NK killing. Primary B6 dendritic cells were observed to become sensitive to killing by autologous NK cells after treatment with emetine. The conclusion that sensitivity to NK killing was attributable to dissociation of preexisting Qa-1\textsuperscript{b}/Qdm complexes was supported by the observation that cytotoxicity of emetine-treated dendritic cells was inhibited by providing an exogenous source of Qdm. Moreover, emetine-treated B6 dendritic cells did not display increased susceptibility to NKG2A\textsuperscript{+} NK cell-mediated lysis. Thus, enhanced cytotoxicity was not due to loss of cell surface Qa-1\textsuperscript{b} molecules in the absence of protein synthesis, but rather to a specific loss of Qa-1\textsuperscript{b}/Qdm peptide complexes.

Overall, our results support the hypothesis that Qa-1 has been selected as a specialized MHC molecule that is unable to form highly stable peptide complexes. We propose that this property has two important consequences related to the function of Qa-1 as a ligand for CD94/NKG2A NK inhibitory receptors. First, the low basal stability of Qa-1-peptide complexes means that only peptides with near ideal anchor residues will bind Qa-1 to form complexes that are stable enough to survive transport and expression at the cell surface. This limits the diversity of peptides presented by Qa-1, such that the repertoire of presented peptides is dominated by Qdm, which possesses an ideal Qa-1 binding motif and is recognized by CD94/NKG2A. Secondly, the relatively rapid dissociation of Qdm from cell surface Qa-1 molecules means that a continuous supply of newly synthesized Qa-1/Qdm complexes is required to maintain a threshold of inhibitory ligand at the cell surface to prevent killing by NK cells. Under conditions in which viruses inhibit protein synthesis, TAP function, or any of the other steps in the general MHC class I biosynthesis pathway required to generate Qa-1/Qdm complexes, infected cells should rapidly become sensitive to killing by CD94/NKG2A\textsuperscript{+} NK cells. Thus, we propose that the CD94/NKG2A-Qa-1/Qdm recognition system has evolved as a rapid sensor of the integrity of the MHC class I biosynthesis and Ag presentation pathway.

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References


