Peptide Specificity of RT1-A1c, an Inhibitory Rat Major Histocompatibility Complex Class I Natural Killer Cell Ligand*

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The rat major histocompatibility complex class Ia allele polymorph RT1-A1c is a potent ligand for the recently identified inhibitory rLy-49 receptor, STOK-2. With the ultimate objective of studying the interactions of these molecules using structural and functional methods, we undertook a detailed study of its peptide specificity. The study revealed that designing an “ideal peptide” by choosing the most abundant residues in the “binding motif” obtained by pool sequencing does not necessarily yield an optimal binding peptide. For RT1-A1c, as many as four positions, P2, P4, P5, and P9, were detected as putative anchors. Since this molecule displays a preference for highly hydrophobic peptides, we tested binding of peptides derived from the known leader peptide sequences of other rat histocompatibility complex class I molecules. One such peptide, found to bind well, requiring 1.6 µM peptide to achieve 50% stabilization, was searched for in vivo. Natural RT1-A1c binding peptides were purified from rat splenocytes and characterized by mass spectrometry using a combined matrix-assisted laser desorption ionization/time-of-flight and quadrupole time-of-flight approach. Results showed that the signal sequence-derived peptide was not detectable in the purified peptide pool, which was composed of a complex spectrum of peptides. Seven of these self-peptides were successfully sequenced.

Many genes of the major histocompatibility complex (MHC) encode proteins that integrate to provide a system for presenting processed self- and non-self-peptide fragments to the cytotoxic CD8+ T-lymphocytes. These antigenic peptides, generated principally by the proteasome, are transported into the endoplasmic reticulum via the heterodimeric transporter associated with antigen presentation (TAP) (1, 2). In the endoplasmic reticulum, some of these peptides associate with newly synthesized MHC class I heavy chains and β2-microglobulin to form complexes, which are then transported through the Golgi network to the cell surface for presentation to the immune system. In recent years, it has become increasingly apparent that cytotoxic CD8+ T-lymphocytes are not the only cell types that recognize class I MHC molecules. Natural killer (NK) cells, another lymphocyte subset in the mammalian immune system, are now known to play important roles in both “innate” immunity and in the regulation of the adaptive immune response. They are early entrants into cellular inflammatory sites, where they can kill virally infected or neoplastic cells and release cytokines, notably substantial quantities of interferon-γ. There is increasing evidence that they can play an important role in the response to and rejection of transplants, notably of bone marrow cell transplants (3, 4), but also of solid organ allo- and xenografts (5). Recent studies of the mechanisms by which NK cells are regulated have led to the discovery of families of NK cell surface receptors that either inhibit (killer inhibitory receptor) or activate (killer activatory receptor) NK cells via recognition of MHC class I molecules. On human NK cells, these receptors are members of the immunoglobulin superfamily, whereas on rodent NK cells the same functions are served by Ly-49 receptors, which are homodimers of the C-type lectin family (6, 7).

The peptide dependences of class I MHC interactions with both types of receptors have been studied, and both were found to interact with the peptide binding α1/α2 domains of the class I molecule (8, 9). In humans, studies have suggested that killer inhibitory receptors can recognize peptide-loaded and -unloaded HLA-C ligands (10) and that different residues at positions 7 and 8 of peptides bound to HLA-B*2705 can influence the interaction between the class I complex and killer inhibitory receptor (11, 12). Experiments with the mouse Ly-49A receptor found that recognition was affected by the absence of peptides but not by the presence of different peptides, thus suggesting recognition was peptide-dependent but not peptide-specific (13). However, this is not the case for every mouse NK receptor, as recent studies with another mouse NK cell receptor, Ly-49I, have shown that it is able to discriminate peptides bound to H-2Kd (14).

Such complexity of interactions seen in both mouse and humans should also be expected in the rat. Although some rat NK cells also recognize classical (RT1-A region-encoded) class I MHC molecules with inhibitory receptors (15–17), a unique feature only described in the rat is that some NK cells are specifically activated by certain polymorphic non-classical
Numerous infectious disease studies have amassed this kind of class I and II in species other than human and mouse where by the lack of known specific peptides able to bind to this was shown to inhibit rat NK cell activity via interaction with a saline containing 0.5% BSA and resuspended in 100

(anti-RT1-A1c). For the second stage antibody, fluorescein isothiocyanate was diluted with Dulbecco’s modified Eagle’s medium and 0.5%

molutions were determined using the micro-BCA assay (Pierce). Each peptide was assayed a minimum of three times, and results are presented as means of the concentrations of peptide required to achieve 50% of the level of cell surface expression after induction at 26 °C (C50).

Relative efficiencies of binding compared with the ideal peptides are illustrated as stabilization index (SI) values on a logarithmic scale, which were calculated as follows: SI = C50(ideal peptide)/C50(ideal peptide).

Immunoprecipitation and Purification of Natural Peptides—The monoclonal antibody YR5/310 (anti-RT1-A1c; Ref. 25) was purified using a Prosep-G column (Bioprocessing Ltd.) and coupled to cyanogen bromide-activated Sepharose (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Splenocyte cell suspensions were made from 20 spleens of FVG.R20 (RT1-A1/TAP2-B) rats. The cell suspension was centrifuged and the pellet washed with buffer A (30 mM Tris, pH 7.7, 150 mM NaCl). The cell pellet was resuspended in 50 ml of buffer A before addition of 50 ml of buffer B (10 mM Tris-HCl, pH 7.7, 150 mM NaCl, 2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). The suspension was mixed at room temperature for 30 min and then spun at 20,000 × g for 30 min at 4 °C. The supernatant was decanted into a fresh bottle and pre-cleared by addition of 3 ml of Sepharose coupled with non-immune rat serum. This was mixed for 1 h at 4 °C before the matrix was removed by centrifugation or filtration. YR5/310-Sepharose (1.5 ml) was then added to the lysate, and the suspension was mixed at 4 °C for another 90 min. The Sepharose was collected and washed extensively with buffer A/B (50:50 v/v), followed by buffer A only. Peptides were eluted by incubating the antibody-coupled Sepha-

rose beads in 1% 10% acetic acid for 5 min at room temperature. After filtration to remove the Sepharose, the solution was passed through Centriprep-3 units, concentrated, and the peptides separated by reverse phase chromatography using an Applied Biosystems Aquapore (250 mm × 1 mm) Brownlee C18 column with an acetonitrile gradient (0–90%) in 0.025% trifluoroacetic acid. Peptides eluted between 6.75% and 40.5% acetonitrile were collected as 60 42-μl fractions and analyzed by mass spectrometry.

Analysis of Natural Peptides by MS—Fractions were examined by MS using a three-step strategy to optimize the sensitivity of the analysis for both peptide detection and sequencing. Initial screening for peptide masses and sample heterogeneity was performed by means of matrix-assisted laser desorption ionization (MALDI) using a Voyager (PerSeptive Biosystems, Framingham, MA) matrix-assisted laser desorption mass spectrometer equipped with a 337-nm nitrogen laser (PE Biosystems, Framingham, MA). A 0.8-μl aliquot (approximately 2% total sample) of each fraction was mixed with 0.8 μl of a 7 mg/ml solution of α-cyano-4-hydroxycinnamic acid (Alrlich) in water/acetonitrile (1:1) incorporating 0.1% trifluoroacetic acid. A 0.5-μl aliquot of this mixture was deposited and allowed to crystallize on the surface of a stainless steel target.

Fractions were then dried under a gentle stream of nitrogen until approximately 1 μl remained and reconstituted to a 4-μl volume with methanol/water (1:1) incorporating 0.1% formic acid (electrospray solvent). Tandem MS with low energy collision-induced dissociation was employed to determine primary sequence using a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, United Kingdom). A 1.5-μl aliquot (approximately 40% of total sample) was introduced into a gold-coated borosilicate capillary with a 1-μm tapered tip (Micromass) for low flow rate electrospray. Argon was employed as the collision gas at an indicated manifold pressure in the analyzer of 6 × 10−5 mbar. Quadrupole resolution was set to give a 4 m/z unit precursor ion window; the product ion resolution was set to 5000 (full width, half-maximum definition). The collision offset was used 18–28 V for doubly charged species.

An additional 1.5-μl aliquot was diluted to 4 μl, and 3 μl of acetic anhydride (Sigma) was added to acetylate the N terminus and the ε-amine of lysine residues. The reaction was left for 15 min at room temperature, dried to approximately 1 μl, and reconstituted in approximately 2 μl of electrospray solvent. A 1.5-μl aliquot was then analyzed on the Q-TOF.

RESULTS

Design of Synthetic Peptides—Peptide elution data for RT1-A1 and RT1-A1α have been published previously (22) and were used to produce ideal peptides based on the most abundant elution for each position (Fig. 1). The following sequences of peptides were deduced and synthesized: NPRKVTAYL (RT1-A1α) and SQFPVSSER (RT1-A1α). For stabilization assays using the TAP2-deficient mouse cell line RMA-S transfected with either RT1-A1c or RT1-A1a, the ideal peptide for one MHC class I molecule was used as a negative control for the other. It was found that, for RT1-A1α, a mean of 604 nm (n = 3) of ideal

(18.5 region-encoded) class I MHC molecules (18). Recently, interest in the RT1-A1c class Ia allotype has increased since it was shown to inhibit rat NK cell activity via interaction with a Ly-49 lectin-like receptor, recognized by the antibody STOK2 (16, 19–21). However, any structural studies on the effect of peptides on RT1-A1/NK cell interactions have been hindered by the lack of known specific peptides able to bind to this molecule. Indeed, this is a general difficulty in studies of MHC class I and II in species other than human and mouse where numerous infectious disease studies have amass this kind of information. In order to address this problem, we have pursued two approaches for the identification of binding peptides. First, based on the previously published binding motif of RT1-A1c (22), peptides were designed, synthesized, and tested for binding to RT1-A1c when expressed in the TAP2-deficient mouse cell line, RMA-S. Second, studies were performed in order to identify natural peptides presented by RT1-A1c on rat splenocytes. Peptides bound to RT1-A1c were isolated by immunoprecipitation, purified by acid elution, and subjected to sequence analysis by mass spectrometry.

For the non-classical class I molecule HLA-E in human and Qa-1b in mouse (23) (and also presumably the Qa-1 homologue, RT-BM1, in rat; Refs. 24 and 25), surface expression is correlated with NK cell inhibition (26–28) and signal peptides derived from MHC class Ia molecules have been shown to be the major peptides bound (23, 29, 30). Our initial examination of the peptide-binding motif of RT1-A1c (22) suggested that this class Ia molecule would bind to some class I signal sequence-derived peptides. Therefore, we performed a limited study to determine directly the ability of RT1-A1c to bind peptides derived from known leader peptide sequences of other rat MHC class I molecules and to determine whether this might have any in vivo significance for NK cell inhibition either directly or indirectly, by affecting the available signal sequence derived peptide thought to be bound to RT-BM1 (known for its mouse and human homologues).

EXPERIMENTAL PROCEDURES

Cell Lines—The preparation of RMA-S cells expressing the RT1-A1 and RT1-A1α molecules has been described elsewhere (31–33).

Synthesis of Peptides—All synthetic peptides were purchased from ECHAZ Microcollections (Tübingen, Germany), except for the rat MTA peptide, ILFPSSRLLR (34), and NPRAMQALL, which were synthesized by Alta Biosciences (University of Birmingham, Birmingham, United Kingdom). For each peptide, the solid was dissolved in 200 μl of Me2SO (Pierce, packed under nitrogen) and stored at −20 °C. Peptide concentrations were determined using the micro-BCA assay (Pierce).

Peptide Stabilization Assays—Peptide stocks, sufficient for the assay, were diluted with Dulbecco’s modified Eagle’s medium and 0.5% (w/v) BSA to give starting concentrations of 100 μM. Serial 5-fold dilutions were made, resulting in six peptide concentrations for each different peptide.

RMA-S cells transfected with the rat MHC class Ia molecules RT1-Aα or RT1-A1α (33) were incubated at 26 °C for 24–48 h to allow expression of surface class I molecules. On the day of the stabilization assay, cells were washed once with cold Dulbecco’s modified Eagle’s medium, 0.5% BSA and resuspended in a volume of the same buffer to give approximately 3 × 106 cells/ml. Cells (40 μl) were aliquoted to a 96-well plate, and 40 μl of peptide solution were added to the appropriate well (highest final concentration of peptide was 50 μM). The “ideal peptide” for one MHC class I molecule was used as a negative control for the other. The plate was incubated at 26 °C for 1 h and then placed in a 37 °C incubator for a further 3 h. Cells were washed once with phosphate-buffered saline containing 0.5% BSA and resuspended in 100 μl of phosphate-buffered saline, 2% (v/v) fetal calf serum, 0.05% (v/v) sodium azide. Peptide-pulsed cells were then subjected to FACS analysis with first stage anti-rat monoclonal antibodies: MAC 30 (anti-RT1-Aα) or YR5/310 (anti-RT1-A1c). For the second stage antibody, fluorescein isothiocyanate-conjugated rabbit anti-rat immunoglobulin (DAKO A/S, Glostrup, Denmark) was used (1/100 dilution). The levels of stabilized rat class I molecules were determined using a FACS Calibur™ (Becton Dickinson). Each peptide was assayed a minimum of three times, and results are presented as means of the concentrations of peptide required to achieve 50% of the level of cell surface expression after induction at 26 °C (C50).

Relative efficiencies of binding compared with the ideal peptides are illustrated as stabilization index (SI) values on a logarithmic scale, which were calculated as follows: SI = C50(ideal peptide)/C50(ideal peptide).
peptide was required to achieve 50% stabilization ($C_{50}$), whereas for RT1-A1c the mean $C_{50}$ was determined as 76 nM, which was about 10-fold better affinity for this peptide-class I combination compared with RT1-A*$^a$ (Fig. 2).

Effect of Anchor Residue Combinations on RT1-A*$^a$ and RT1-A1c Stabilization—With a 10-fold discrepancy in affinity between these two class I molecules with their respective ideal peptides, further combinations of anchor residues for RT1-A*$^a$ were tested. For this particular study, we tested an arginine substitution at P1, leucine and methionine at P2, and an asparagine at P8. These residues were all seen as increases in peptide affinity. At each position of the ideal peptide, residues with the largest percentage increase were selected. In the P1 position, the amino acid with the greatest yield was selected. If no increase was observed at a particular cycle, a serine was selected.

Table I gives a summary of the fractions sampled and the peptide sequences deduced. Initially, fractions were analyzed by MALDI/MS; low commitment of sample (~2%) with good signal/noise ratios and the observation of only singly charged ions made this an invaluable screening process. Fraction 32 revealed an ion at m/z 1013.54. Preliminary knowledge of peptide masses enabled investigation of subsequent electrospray MS spectra that, in the presence of co-eluting peptide, gave poor signal/noise ratios. High sensitivity tandem MS experiments were possible using low flow rate electrospray Q-TOF when ions in the MS spectra were poorly observed within chemical noise. Fig. 6 (top spectrum) shows the products of an ion at m/z 507.3, [M + 2H]$^{2+}$, giving a dominant b-ion series (corresponding to peptide bond cleavage with charge retention on the N-terminal fragment). Following N-acetylation (which allows distinction between isobaric lysine, glutamine, and b-ion identification) and subsequent re-analysis (Fig. 6, bottom spectrum) the sequence NPRAMQALL/I/L/I was deduced. Leucine/isoleucine distinction was not possible by this method, so in order to assess the effects of leucine/isoleucine P8 and P9 variations on binding to RT1-A1c, the four possible variants of the peptide (-II, -IL, -LI, and -LL) were synthesized and tested using low flow rate electrospray Q-TOF when ions in the MS spectra were poorly observed within chemical noise. In Fig. 7 (left spectrum) shows the products of an ion at m/z 507.3, [M + 2H]$^{2+}$, giving a dominant b-ion series (corresponding to peptide bond cleavage with charge retention on the N-terminal fragment). Following N-acetylation (which allows distinction between isobaric lysine, glutamine, and b-ion identification) and subsequent re-analysis (Fig. 7, bottom spectrum) the sequence NPRAMQALL eluted in fraction 32, and subsequent tandem MS analysis of the synthetic peptide (data not shown) confirmed the deduced sequence.

Peptide sequences were determined using a three-stage strategy; the analysis of a component in fraction 32 is described. Initially, fractions were analyzed by MALDI/MS; low commitment of sample (~2%) with good signal/noise ratios and the observation of only singly charged ions make this an invaluable screening process. Fraction 32 revealed an ion at m/z 1013.54. Preliminary knowledge of peptide masses enabled investigation of subsequent electrospray MS spectra that, in the presence of co-eluting peptide, gave poor signal/noise ratios. High sensitivity tandem MS experiments were possible using low flow rate electrospray Q-TOF when ions in the MS spectra were poorly observed within chemical noise. Fig. 6 (top spectrum) shows the products of an ion at m/z 507.3, [M + 2H]$^{2+}$, giving a dominant b-ion series (corresponding to peptide bond cleavage with charge retention on the N-terminal fragment). Following N-acetylation (which allows distinction between isobaric lysine, glutamine, and b-ion identification) and subsequent re-analysis (Fig. 6, bottom spectrum) the sequence NPRAMQALL/I/L/I was deduced. Leucine/isoleucine distinction was not possible by this method, so in order to assess the effects of leucine/isoleucine P8 and P9 variations on binding to RT1-A1c, the four possible variants of the peptide (-II, -IL, -LI, and -LL) were synthesized and used in the RMA-S stabilization assay. Results (not shown) revealed that all four peptides bound similarly, requiring a concentration of less than 20 nM to achieve 50% stabilization. Because these results were close to the lowest peptide concentration tested (16 nM), no distinction could be made between Leu/Ile preference at either position. A search of the data bases with these four possible peptides identified NPRAMQALL as part of a rat protein found associated with a candidate tumor suppressor protein (accession no. D87950) (36). Homologues of this protein were also identified in both humans (accession no. AB113544) (37) and Xenopus (accession no. AB030502) (38), indicating conservation of this protein across species. Reverse phase chromatography of the synthetic peptide NPRAMQALL eluted in fraction 32, and subsequent tandem MS analysis of the synthetic peptide (data not shown) confirmed the deduced sequence.
shown) showed that peptides binding to RT1-A1c with Leu in the P9 position were preferred over Ile, whereas in the P6 position Ile was preferred over Leu. The best peptide found to bind was, therefore, DPSQHIVQL (C50 539 nM), although all other peptides tested were still in the submicromolar range. All data base searches were unsuccessful with any Leu/Ile combination.

For the remaining results in Table I, the majority of partial peptide sequences conform to the previously published binding motif (22). Data base searches of both full and partial results revealed only two other possible hits. For the N(L/I)PPH(L/I)(L/I)R(L/I) peptide from fraction 35, the peptide NLPPHIIRL, from a ubiquitin-conjugating enzyme (residues 7–15, m/z 1073.3) is present (Swissprot code UBCE_HUMAN), and a rat homologue for this gene is also described in the expressed sequence tag data bases (accession no. H33742). For the peptide NP(RS/SR)WEVV(L/I), a mouse expressed sequence tag (accession no. AU079261) exists, which codes for the peptide NPSRWEVVVL; however, its sequence shares no homology with any other data base entry.

Peptide Specificity of RT1-A1c

Comparison of the leader peptide sequences known to date for rat class Ia MHC molecule shows that they all carry the same 11-mer peptide (AMAPRTLLLLL): RT1-A1n (accession no. U50448), RT1-A2c (accession no. U38971), RT1-A1a (accession no. M31018), RT1-A1b (accession no. U38970), RT1-A1u (accession no. U38972), and RT1-A1l (accession no. L26224). Three overlapping nonamer peptides based on this 11-amino acid stretch were synthesized and tested for stabilization to RT1-A1c by the RMA-S stabilization assay. Results (Fig. 7) show that both the peptides AMAPRTLLL and MAPRTLLLL bound poorly to RT1-A1c in this assay (C50 stabilizations of 35 and 15 μM, respectively). However, the third peptide tested (APRTLLLLL), which fits the binding motif, bound best with a C50 stabilization of 1.6 μM, only 20-fold lower than the ideal peptide. In order to determine whether this peptide was processed in the rat in vivo, the synthetic peptide was subjected to the same reverse phase chromatography conditions used for purifying natural RT1-A1c binding peptides from rat splenocytes. The peptide eluted with a retention of 6.3–6.4 ml, corresponding to fractions 48–49 of the elution profile shown in Fig. 5 (results not shown). MALDI/TOF analysis of these and surrounding fractions (fractions 46–51), however, revealed no peptide of the expected mass [(M+H)+ = 1010.3], suggesting that in this experiment, such a peptide was not naturally present in detectable amounts. No other peptide variants based on the known signal sequences were searched for in this study.
RT1-A1c (C50 assay with a mean C50 of 540 nM (Fig. 2). Therefore, any lymphocytes can be generated (34) and which binds in this shows comparable affinity to the natural 13-mer mitochondrial (NPRKVTAYL) was designed on the basis of the previously molecules was determined by FACS analysis. An ideal peptide solutions of specific peptides, and their ability to stabilize the achieved. Such molecules were then incubated with serial di-

ecule, RT1-Aa, was also designed from its binding motif (Fig. 1 this approach, an ideal peptide for a second class I MHC mol-

lized. In order to control the validity of each position of the peptide. In order to control the selectivity of the F pocket isoleucine substitution produced a 3-fold reduction in binding. This indicates a high level of selectivity for Leu by the F pocket. Changes at the P2 position and Asn at the P8 position, whereas a synergistic effect was observed when substitutions occurred at both positions (10-fold increase). Therefore, in the case of RT1-Aa, choosing the most abundant residue at each position did not produce the tightest binder, suggesting that caution must be exercised in any conclusions drawn from sequencing pooled peptide populations. For RT1-Aa, the pool sequencing data (22) revealed increases for Gln (39%), Leu (31%), and Met (30%) seen at P2. Values in parentheses are percentages of the total amino acid yield for these three residues at cycle 2. Yield variations from one experiment to another could easily change these values. In addition, amino acid contamination of the first sequencing cycle can easily reduce the calculated percentage increases of certain residues at the P2 position. However, such contamination was unlikely to influence the peptide design for RT1-A1c where proline was 69% of the yield at the P2 position (22).

As was done for RT1-Aa, single substituted variants of the ideal peptide for RT1-A1c were made and tested for binding to the RMAS-A1c cell line. Of the 32 peptides synthesized, only 5 were significantly better binders and the improvement in binding affinity was always less than 2-fold (see Fig. 2). The majority of peptides tested exhibited reduced binding affinity. Results revealed that changes at the P1 and P3 positions had little effect on peptide binding, whereas the P2 position appeared unable to accommodate charged residues, particularly aspartic acid (C50 = 1.32 μM). In addition, all except one of the residue changes in the P4, P5, P8, and P9 positions reduced binding affinity. Such an intolerance to single residue substitutions suggests that there are peptide side chain/MHC interactions along the length of the peptide. This would be consistent with previous studies of peptide length preferences for this molecule, which found a restricted preference for peptides of 9–12 amino acids in length (33). The greatest effect was seen at the P9 position, where both charged and aromatic substitutions resulted in a 10–100-fold reduction in peptide binding. Even an isoleucine substitution produced a 3-fold reduction in binding. This indicates a high level of selectivity for Leu by the F pocket in the binding groove and consequently the importance of the side chain of the C-terminal residue for peptide stability.

For the second approach, studies were performed in order to identify natural peptides presented by RT1-A1c on rat splenocytes. Peptides bound to RT1-A1c were isolated by immunoprecipitation, purified by acid elution and reverse phase chromatography, and subjected to sequence analysis by mass spectrometry. A sample (0.5 μl) from each fraction was pooled and analyzed by MALDI/MS to determine the range of peptide populations. For RT1-Aa, the pool sequencing data (22) revealed that proline was the most abundant residue at the P2 position (69%).

**DISCUSSION**

Here, we have adopted two approaches for the identification of peptides that will bind to the rat MHC class Ia allotype RT1-A1. The first approach was to design, synthesize, and test peptides for binding to RT1-A1 when expressed in the TAP2-deficient mouse cell line, RMA-S. By incubation of this cell line at 26 °C, expression of “peptide-empty” complexes could be achieved. Such molecules were then incubated with serial dilutions of specific peptides, and their ability to stabilize the molecules was determined by FACS analysis. An ideal peptide (NPRKVTAYL) was designed on the basis of the previously published peptide elution data for RT1-A1 (Fig. 1 and Ref. 22), using the most abundant residues in the eluted peptide pool at each position of the peptide. In order to control the validity of this approach, an ideal peptide for a second class I MHC molecule, RT1-Aa, was also designed from its binding motif (Fig. 1 and Ref. 22) and synthesized, since the RMA-S cell line transfected with RT1-Aa was also available (31, 32). Results showed that for this control class I MHC molecule, the ideal peptide designed (SQPPVSERR) bound with almost 10-fold reduced affinity (C50 = 604 nM) compared with the peptide designed for RT1-A1c (C50 = 76 nM). However, in the context of natural peptides, the RT1-Aa ideal peptide is still a good binder as it shows comparable affinity to the natural 13-mer mitochondrial minor histocompatibility antigen against which cytotoxic T lymphocytes can be generated (34) and which binds in this assay with a mean C50 of 540 nM (Fig. 2). Therefore, any natural peptide stabilizing in this system with a C50 < 1 μM might well be found in vivo.

To determine if the observed weak binding was due to peptide design, variants of the peptide with single di- or tri-substituted residues were synthesized and their binding affinity was also assessed. Results in Fig. 2 show clearly that substitution of an arginine at the P1 position has a deleterious effect on peptide binding. However, improved binding (2-fold increase) was observed when either Leu or Met were substituted at the P2 position and Asn at the P8 position, whereas a synergistic effect was observed when substitutions occurred at both positions (10-fold increase). Therefore, in the case of RT1-Aa, choosing the most abundant residue at each position did not produce the tightest binder, suggesting that caution must be exercised in any conclusions drawn from sequencing pooled peptide populations. For RT1-Aa, the pool sequencing data (22) revealed increases for Gln (39%), Leu (31%), and Met (30%) seen at P2. Values in parentheses are percentages of the total amino acid yield for these three residues at cycle 2. Yield variations from one experiment to another could easily change these values. In addition, amino acid contamination of the first sequencing cycle can easily reduce the calculated percentage increases of certain residues at the P2 position. However, such contamination was unlikely to influence the peptide design for RT1-A1c where proline was 69% of the yield at the P2 position (22).

FIG. 4. Effect of single residue changes on the stabilization of the ideal peptide on the rat class Ia molecule, RT1-A1c, when expressed on the surface of RMA-S cells. Experiments were performed as for RT1-Aa before assessing successful stabilization by FACS analysis. Relative efficiencies of binding compared with the ideal peptide are illustrated as stabilization index (SI) values on a logarithmic scale. (For calculation, see “Experimental Procedures.”) Compared with the results of the ideal peptide (NPRKVTAYL), statistically significant results are marked with single asterisk (**; p < 0.05) and double asterisks (**; p < 0.001).
Of the peptide species analyzed, only seven full sequences were successfully determined. There were two major reasons. First, detergent carry-over from the immunoprecipitation step prevented successful analysis of many fractions. Here, preferential ionization of the detergent by electrospray resulted in low peptide ion yields; co-selection of detergent ions with peptides for low energy collision-induced dissociation also complicated tandem MS data. Second, the presence of a mid-chain arginine residue appears to reduce the efficiency of cleavage of the peptide backbone at proximal sites. This reduction was observed with six of the seven fully sequenced peptides, the exception not containing arginine. The consequent reliance on low intensity signals for determination of portions of each sequence prevented full structure determination using the lower intensity product ion spectra recorded for other examples. Such peptides yielded either high member y- or b-ion series only.

Among the seven full sequences, only three revealed hits in the data bases, of which two can be attributed to known proteins. Both peptides originate from proteins with probable high rates of turnover. Consequently, such proteins would be expected to provide peptides in sufficient amounts to be detected. As the peptides under analysis were of rat origin, it is not surprising that few positive identifications were possible. Sequence information from the rat is more limited than that from either mouse and human. It is fortunate that both the identified peptides were derived from sequences conserved across species. This appears not to be the case for the other five full sequences identified in this study, although they may be components of as yet unknown conserved proteins.

For the human non-classical class I molecule HLA-E and its counterpart Qa-1b in mouse (23) (and presumably RT-BM1 in the rat), surface expression is correlated with NK cell inhibition (26) and the presentation of signal peptides derived from classical MHC class Ia molecules (25, 29, 30). The mechanism for the delivery/assembly of signal peptides to HLA-E was shown to be TAP-associated (39), although it has not been unequivocally proven that the TAP transporter is responsible for delivery of these peptides from the cytoplasm. If such peptides are indeed supplied by the TAP transporter, then they represent only a very small fraction of the total peptide pool transported, while a much larger fraction of this pool will end...
up bound to other more common classical class Ia molecules and not HLA-E. Issues that may need to be addressed include: (i) what role, if any, do these other classical class Ia molecules play in sequestering signal peptides from HLA-E associations; (ii) what proteolytic pathways for processing these signal peptides are in operation within the cell, and (iii) are there specialized mechanisms for the delivery of these peptides for binding to HLA-E? Previous work suggests that all three possibilities might operate. Two groups (40, 41) found that HLA-A2 molecules in the human TAP transporter-defective T2 cell line bound a limited set of endogenous peptides derived from signal sequences, thus suggesting that such peptides might reach the endoplasmic reticulum lumen directly, via a TAP-independent route. One of the described peptides has also been found bound to HLA-A2 in the non-mutant cell line, C1R-A2.1 (42). These results also revealed peptides derived from the same signal sequence but of different lengths, suggesting that proteolytic systems operate to process these peptides into products of variable lengths. Finally, evidence exists to suggest that other classical class I molecules might associate with signal peptides derived from all three classes of MHC molecules: classical class I (43), non-classical class I (42, 44), and class II (43).

Consequently, we have investigated the ability of RT1-A1c to bind peptides derived from known leader peptide sequences of other rat MHC class I molecules since this might have in vivo significance for direct NK cell inhibition or act indirectly by affecting the available signal sequence derived peptide thought to be bound to the rat HLA-E homologue, RT-BM1. Results showed that the rat classical class Ia molecule RT1-A1c exhibited a low affinity for binding the leader-derived peptide, AMA-PRTLLL (C50 of 35 μM), but had a higher affinity for the overlapping peptide, APRTLLLLL (C50 of 1.6 μM; see Fig. 7). If

| Fraction no. | m/z [M+H]+ | Deduced sequence | Origin |
|-------------|------------|-----------------|--------|
| 21          | 1008.6     | D.PS.QV--       | Unknown|
| 21          | 1081.6     | ==EHTA (L/I) (L/I) | Unknown|
| 21          | 1103.6     | NPHQ--          | Unknown|
| 21*         | 1145.6     | NHPL (L/I) GE (L/I) PR (L/I) | Unknown|
| 24          | 1032.6     | -- (L/I) HPV (L/I) | Unknown|
| 24*         | 1036.5     | DPSQH (L/I) VQ (L/I) | Unknown|
| 24          | 1074.6     | VFR=DPT (L/I) | Unknown|
| 27          | 981.4      | -- (L/I) SHV (L/I) | Unknown|
| 27*         | 1022.4     | --VF (L/I) (L/I) | Unknown|
| 27*         | 1077.4     | NVAQDYVR (L/I) | Unknown|
| 30          | 808.4      | PSP--           | Unknown|
| 30          | 907.4      | VPS--           | Unknown|
| 30          | 1000.6     | VPS--F (L/I) | Unknown|
| 30          | 1035.6     | R (L/I) -- (L/I) (L/I) (L/I) | Unknown|
| 32*         | 1013.4     | NFRAMQ (L/I) (L/I) | Protein associated with tumor-suppressor protein/putative transcription factora|
| 35          | 1045.6     | NP-- (L/I) F (L/I) | Unknown|
| 35*         | 1073.1     | N (L/I) PPH (L/I) (L/I) R (L/I) | Ubiquitin protein ligaseb|
| 36          | 1040.3     | NPY--           | Unknown|
| 38          | 1002.6     | QPAS--FD (L/I) or NF--FD (L/I) FD (L/I) | Unknown|
| 38*         | 1090.7     | NHLD (L/I) (L/I) R (L/I) (L/I) | Unknown|
| 38*         | 1099.6     | NP(RS/SR)WEVV (L/I) | Unknownc|
| 41          | 1056.8     | D--NV (L/I) (L/I) | Unknown|

Fig. 7. Stabilization of signal peptide-derived peptides on RT1-A1c. Three overlapping nonamer peptides covering an 11 residue stretch of a rat MHC class I signal peptide were tested for stabilizing RT1-A1c on RMA-S cells. Results are presented as the percentage of stabilization relative to cells maintained at 26 °C and compared with stabilization curves with the ideal peptides of RT1-A1c (positive control) and RT1-Aa (negative control). Results are presented as the mean of three independent experiments.

Table I. Sequences of natural peptides associated with RT1-A1c deduced by MS

Tandem MS with low energy collision-induced dissociation (CID) was employed to determine primary sequence using a quadrupole time-of-flight (Q-TOF) mass spectrometer. Lys and Gln residues were assigned by analysis before and after acetylation (see "Experimental Procedures"), whereas Leu and Ile could not be distinguished. Full sequences deduced are indicated by an asterisk (*).
classical class 1a molecules can bind these leader-derived peptides and thereby prevent their binding to RT-BM1 (and its homologues), this could increase the requirement for specific delivery of these peptides to the RT-BM1 assembly complexes. In addition, the observation that the peptide APRTL{L}L{L}LL was a better binding peptide for RT1-A1c also suggests the possibility that class I leader-peptides might be processed by different routes/proteases to derive such peptides. If this occurs, then there might be even less material available for processing and binding to RT-BM1. Interestingly, the corresponding HLA-A2.1 signal peptide (APRT{L}VL{L}L) has been identified bound to the human classical class I molecule HLA-B7 (43), suggesting alternative proteolytic processing. In order to determine this possibility in the rat in vivo, we looked for a peptide of the expected mass for APRTL{L}L{L}LL in the purified RT1-A1 c binding peptides from rat splenocytes. MALDI/TOF analysis was negative, suggesting that, in this experiment, such a peptide was not present in detectable amounts. A more detailed study encompassing the other known rat classical class I molecules and other fragments of the signal peptide would be needed before a full conclusion could be drawn.

In summary, we have identified natural and synthetic RT1-A1c binding peptides. One of these, NPR{A}MQ{A}LL, has already enabled us to obtain crystals of RT1-A1c peptide complexes with excellent x-ray diffraction characteristic (work in progress by Dr. J. Spier and Prof. I. A. Wilson, Scripps Research Institute, San Diego, CA). The peptide identified will allow future studies to probe the effect of peptide variants on possible NK cell interactions using both biophysical and cell-based assays. The approaches to the design of synthetic peptides described here, combined with the method for elucidating peptide binding motifs that we have recently described (22), can greatly assist the identification of high affinity peptides for any given class I molecule.

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