The use of synthetic random peptide libraries is a powerful technology for the study of many aspects of antigen presentation and peptide selection by major histocompatibility complex (MHC) molecules. Here we have used them in conjunction with a recombiant system to determine the peptide binding motifs of three classical class I MHC molecules of the laboratory rat: RT1-Aa, RT1-Au, and RT1-A1c. Described is a method for producing large amounts of soluble class I heavy and light chains in bacteria. Refolding RT1-Aa heavy chain (HC) with rat β2-microglobulin (β2m) in the presence of a specific peptide and the subsequent purification of the complex yielded conformationally correct material. This was assessed by gel chromatography, SDS-polyacrylamide gel electrophoresis, isoelectric focussing gel electrophoresis, isolectric focusing gel electrophoresis, enzyme-linked immunosorbent assay, and fluorescence-activated cell sorter analysis employing a previously unreported method utilizing a His-Tag affinity silica. By refolding RT1-Aa HC and rat β2m around a random nonapeptide library and subjecting the resulting complex to acid elution of the bound peptides and pool sequencing, the peptide binding motif for this MHC class I molecule was determined. Results corresponded well with those previously determined from naturally bound peptides and in addition gave a clear and unambiguous signal for the C-terminal anchor residue. This method was then applied to determine the previously undescribed binding motifs for RT1-Au and RT1-A1c. For both molecules, the whole motif was confirmed from naturally bound peptides. We propose this method as an alternative way to obtain the whole class I MHC peptide motif, particularly when a specific antibody is unavailable and/or natural expression of the class I molecule of interest is low.

Antigenic peptides generated principally by the proteasome are transported into the endoplasmic reticulum (ER) via the heterodimeric transporter associated with antigen presentation (TAP) (1, 2) where they associate with newly synthesized major histocompatibility complex (MHC) class I molecules. In the rat, the TAP2 subunit of the transporter exists as two main allotopes (TAP2-A and TAP2-B) (3, 4), and it appears that class I MHC alleles, encoded by the classical class Ia locus RT1-A, are genetically associated with only one form of the transporter (4). In recent years, it has been shown that the two forms of the transporter display different peptide selectivities, such that TAP-A binds and transports peptides with many different amino acids present at the C terminus while TAP-B generally allows only hydrophobic and aromatic residues in that position (5, 6). In addition, the TAP-B form of the transporter has been reported to be able to accept longer peptides than the usual 8–11 amino acid lengths (7) although this is disputed (8).

Despite extensive studies on TAP peptide selectivities, few peptide binding motifs for rat class Ia molecules (RT1-A) have been reported. Thus far, the motifs of only two TAP-A associated molecules, RT1-A1 (9, 10) and RT1-Aa (11), have been determined.

The main method used to determine a peptide binding motif for an MHC class I molecule, including those rat alleles already reported, is the pool sequencing method of Falk et al. (12), that consists of acid eluting peptides from a natural source of class I, separating the heavy and light chains from the peptides by reverse phase HPLC, pooling the predetermined peptide containing fractions, and subjecting the pool to automated sequence analysis. This method has proven extremely useful for obtaining peptide binding data for class I from different species. However, for its success, the method relies on a specific antibody for the purification of the class I under study, but this is not always available. Antibodies can often have cross-reactivities with other known or unknown class Ia or class Ib molecules (13). If expression of the molecule under study is low, then cloning and expression in a suitable cell line is needed. In addition, the C-terminal anchor residue is not always clearly defined by this sequencing method since class I alleles can bind peptides of varying lengths, thus spreading the C-terminal signal over several sequencing cycles (14–16).

An alternative method called positional scanning (17) has been shown to be extremely useful within the field of immunology (18–20). Synthetic random peptide libraries coupled to single defined amino acids at set positions within the peptide have been used successfully to probe the effect of the
defined amino acid on peptide-MHC interactions, induction of T-cell responses, specificity of proteasomes (21), and TAP (22, 23).

Here, we have combined the above methods to determine peptide binding motifs for class I MHC molecules. We report the use of a bacterial system to produce recombinant protein of three truncated rat class Ia MHC molecules and rat β₂m-microglobulin (β₂m). We have successfully refolded these rat class I heavy chains in the presence of rat β₂m and a random non-apeptide library and have successfully determined the binding motifs for all three molecules under study.

EXPERIMENTAL PROCEDURES

Expression and Purification—The regions coding for amino acids 1–278 of the RT1-Aa, RT1-Au, and RT1-A1c heavy chains were cloned from cDNA by polymerase chain reaction (PCR) using the oligonucleotide primers: forward 5′-CCGGATCCCATATGGCGGCTACA-
CTCCGCTGGATT-3′ and reverse 5′-GAAGATCTCGAGCCGCCCTCTACTGCTCCA-
CCCACCTCTGGGAAAGTGGC-3′ (bold are coding nucleotides and underlined are restriction sites used). Full-length rat β₂m was amplified from PVG-RT1-A(α0) poly T-prime reverse strand concana-
valin A plasmids, with primers designed using the nucleotide sequence from the EMBL data base: accession number Y00441, forward 5′-CCGGATCCCATATGGCGGCTACA-
CTCCGCTGGATT-3′ and reverse 5′-GGAATCTCGAGCCGCCCTCTACTGCTCCA-
CCCACCTCTGGGAAAGTGGC-3′ (annals 1 base pairs downstream of the stop codon). The resulting fragments were digested with NdeI/XhoI (heavy chain) and NdeI/BamHI (β₂m), ligated separately into the T7 expression plasmid pET-22b(+) (Novagen Inc., W1), and transformed/selected in XL2-Blue (Stratagene). DNA sequences from these constructs were checked, and plasmids were separately into the T7 expression plasmid pET-22b(+) (Novagen Inc., W1) and submitted for sequencing. As described (11, 12, 33), a pmol yield of 150% of the previous cycle was considered significant and is presented as boxed values in the results (Figs. 1–3). For cycles 7–10, this cut-off value was reduced to 120% to allow for cycle-to-cycle sample loss. For RT1-A1c, because of the increased scale and amino acid yield, the cut-off values used for the random peptide refolding were increased to 300% for cycles 1–6 and 150% for cycles 7–10. Residues at cycle 1 were difficult to assign as anchors, mainly due to having no previous cycle with which to compare and that the first cycle is often prone to amino acid contamination. Amino acids presented at cycle 1 are the 3–4 amino acids with the greatest yield (glycine was ignored due to contamination).

Conformational Antibody Experiments—Successful refolding was determined by ELISA using the conformational RT1-A specific antibody (MAC 30, Ref. 34), a general class I reactive antibody (MRC-OX18, Ref. 25), and a β₂m specific antibody (C9, Ref. 26). The complex to be tested was coated onto plates overnight at 4 °C before addition of antibodies. A horseradish peroxidase-secondary antibody was used, and the plates were developed with TMB and read at 450 nm.

In addition to ELISA, FACS analysis of Ni-NTA silica beads (Qiagen Inc., CA) coated with refolded complexes was used. For coating, 40 μg of the refolded complex was incubated with 50 μg of silica beads pre-equilibrated in PBS, and the mixture was incubated at room temperature for 2 h with gentle mixing to allow the complex to associate to the matrix via the α-chains C-terminal His-Tag. After washing any excess protein away with PBS, 0.1% (v/v) Tween 20, the matrix was incubated with MAC 30, followed by a fluorescein isothiocyanate-coupled antimouse- 
IgG antibody (DAKO A/S, Denmark) at a 1/100 dilution in PBS. After extensive washing with PBS, the matrix was subjected to FACS analysis using a FACS Calibur™ (Becton Dickinson).

Immunoprecipitation Experiments—Monoclonal antibodies U9F4 (anti-RT1-A*, Ref. 35) and YR5310 (anti-RT1-A*, Ref. 13) were purified and coupled to cyanogen bromide-activated Sepharose (Pharmacia) according to the manufacturer instructions. CS8 cells (RT1-A*+TAP2-B) (3) were cultured to approximately one million cells per ml (1.5 liters total volume). Splenocyte cell suspensions were made from 15 spleens of PVR.G20 (RT1-A1c/TAP2-B) rats.

For both cell types, the cell suspension was centrifuged and the pellets were washed with buffer A (10 mM Tris, pH 7.7, 150 mM NaCl). The cell pellets were resuspended in 100 μl of buffer B (10 mM Tris-HCl, pH 7.7, 150 mM NaCl, 2% Nonidet P-40, 1 mM PMSF). The suspension was mixed at room temperature for 30 min and then spun at 20,000 × g for 30 min at 4 °C. The supernatants were decanted into fresh bottles, and 3 ml of non-immune rat serum-Sepharose was added. This was mixed for 1 h at 4 °C before the matrices were removed by centrifugation/filtration. U9F4-Sepharose and YR5310-Sepharose (1.5 ml for both) were then added to their
respective lysates and mixed at 4 °C for a further 90 min. The Sepharose was collected and washed thoroughly with buffer B followed by buffer A. Peptides were eluted by incubating the antibody-coupled Sepharose with 15 ml of 10% acetic acid for 5 min at room temperature. After filtration, the solutions were then passed through Centriprep-3 units and concentrated, and the peptides were separated by reverse phase chromatography using the same method described above for the peptides released from the refolded complex. The cut-off values used to determine binding motifs were a 300% increase over the previous cycle for cycles 1–6 and 150% for cycles 7–10.

RESULTS

Expression and Purification—Using PCR, we have engineered expression plasmids to produce three different soluble rat class I RT1-A molecules in E. coli, RT1-Aa, RT1-Au, and RT1-A1c. Under isopropyl-β-D-thiogalactopyranoside induction, both β2m and the heavy chains were expressed as inclusion bodies and therefore required urea solubilization before further purification could be performed. Induction of the heavy
FIG. 2. Results of pool sequencing of peptides bound to recombinant RT1-A<sup>a</sup> and peptides derived from the C59 cell line. A, experimental details for recombinant RT1-A<sup>a</sup> are as described under “Experimental Procedures” and the legend for Fig. 1. B, RT1.A<sup>a</sup> complexes were isolated by immunoprecipitation using a U9F4-Sepharose matrix, and their bound peptides were purified by acid elution and reverse phase chromatography. Pooled peptides were sequenced by Edman degradation. Anchor residues were determined as described under “Experimental Procedures” and are boxed while other increases are underlined.
chain constructs produced material consistent in size, by SDS-PAGE, with that expected for the truncated, 1–276-amino acid heavy chains coupled at their C-termini to an 8-amino acid stretch containing a His-Tag motif (LEHHHHHH) coded by the pET-22b(+) plasmid. The resulting class I heavy chains were further purified on an Ni-NTA-agarose column to >95% purity.

**Fig. 3.** Results of pool sequencing of peptides bound to recombinant RT1-A1c and peptides derived from PVG.R20 splenocytes. A, experimental details for recombinant RT1.A1c are as described under “Experimental Procedures” and the legend for Fig. 1. B, RT1.A1c complexes were isolated by immunoprecipitation using a YR5/310-Sepharose matrix, and their bound peptides were purified by acid elution and reverse phase chromatography. Pooled peptides were sequenced by Edman degradation. Anchor residues were determined as described under “Experimental Procedures” and are boxed while other increases are underlined.
(Fig. 4). The yield of heavy chain was 10–20 mg/liter of bacterial culture. This eluted off the Ni-NTA column as two species, one in pH 5.9 and the other in pH 4.5 buffer (Fig. 4; last two lanes). SDS-PAGE in the presence/absence of reducing agent confirmed the mixture eluting in the pH 5.9 buffer to be mainly composed of monomers, while protein eluting in the pH 4.5 buffer comprised large molecular weight multimers (results not shown). Only the monomer fraction was used for refolding work (yield 5–10 mg/liter). Protein sequencing of the monomer fraction revealed that the bacteria had successfully cleaved the initiation methionine to give the expected N-termini (results not shown).

The \( \beta_m \) construct was cloned into the plasmid with its own stop codon, thereby producing only the native protein (99 amino acids in length). Successful purification was achieved first by refolding the urea-solubilized material by dialysis against 10 mM Tris-HCl, pH 7.5, and subsequently by ion exchange chromatography of the soluble fraction. Yields could be increased by subjecting the insoluble fraction, produced during dialysis, to a further round of solubilization/dialysis. Ion exchange chromatography of \( \beta_m \) was performed on a Q-Sepharose (HiLoad 16/10) column. Protein (>80% purity) composed of \( \beta_m \) aggregates eluted in 10 mM Tris-HCl, pH 7.5, while the monomer fraction (>95% purity) appeared in the 50 mM NaCl wash (SDS-PAGE results not shown). The yield of \( \beta_m \) was 40–50 mg/liter of bacterial culture of which 20–25 mg/liter were monomers. Only the monomer fraction was used for refolding work. Protein sequencing revealed the expected N-terminus (MIQKT-\( \beta_m \)) but the bacteria had only deformylated and not cleaved the initiation formyl-methionine (results not shown). This is probably due to inhibition of bacterial methionine aminopeptidase by the adjacent isoleucine (first residue of \( \beta_m \)), as discussed by Hirel et al. (36). The methionine, however, should not interfere with subsequent refolding experiments since, in all the reported structures of class I molecules, position 1 is away from the binding groove and the \( \alpha \)-chain/\( \beta_m \) interface (37).

**Specific Peptide Refolding**—Refolding tests were initially performed using the RT1-A\(^a\) heavy chain because, thus far, this is the only rat class Ia molecule for which a specific peptide has been identified and reported to bind (27). The peptide used for refolding was ILFPSSERLSNR, a 13-mer peptide from the rat mitochondrial A chain of ATP synthase (SwissProt accession number P05504). Results from a typical refolding experiment, using a 10-fold excess of peptide over the heavy chain, are shown in Fig. 5A. Gel filtration of the refolding mixture through a Superdex 75 column gave three peaks eluting at >100 kDa (Peak A), ~40 kDa (Peak B), and 12 kDa (Peak C). These corresponded to aggregates, refolded complex, and \( \beta_m \), respectively. Peak B increased in the presence of peptide, and SDS-PAGE analysis showed that there were both heavy chain and \( \beta_m \) bands present (Fig. 5B). When refolding is performed without peptide, the observed peak at ~40 kDa could be an empty heavy chain/\( \beta_m \) complex, but this has yet to be characterized. Isoelectric focussing gel electrophoresis and a subsequent Western blot of the peptide/MHC complex revealed bands with an approximate pI of 6.7 for free \( \beta_m \) (C9 positive) and with pI 5.5 for the heavy chain/\( \beta_m \) complex (MRC-OX18 and C9 positive), and at pI 5.1, two almost indistinguishable bands (MRC-OX18 positive) were revealed that could be heavy chain with/without peptide (results not shown).

To assess whether the purified complex was truly conformational, we performed ELISA assays and FACS analysis. For the ELISA, three antibodies were tested: MRC-OX18 (general anti-rat MHC class I), MAC 30 (anti-RT1-A\(^a\)), and C9 (anti-rat \( \beta_m \)). Fig. 6A shows the results of the ELISA. MRC-OX18 was positive for all forms where the heavy chain was present, consistent with its broad specificity. The reactivity of MAC 30 increased with the addition of peptide and was positive in the purified complex, which was also positive for C9 (anti-\( \beta_m \)).

FACS analysis with MAC 30 on complex bound to Ni-NTA silica recorded an increase in the mean fluorescence (Fig. 6B), further suggesting the purified complex was in its correct conformation. Finally, acid elution of the peptide bound in the complex and sequencing of the eluted material, <3 kDa, gave the expected sequence of the 13-mer peptide.

**Random Peptide Refolding Experiments**—Direct pool sequencing of the random nonapeptide library showed no significant enrichments for any amino acids at any position (results not shown). Therefore, any enrichment seen in the material eluted from the refolded RT1-A molecules was considered specific.

For RT1-A\(^a\), a 10-fold molar excess of random peptide:heavy chain was sufficient to give equivalent successful refolding compared with the specific 13-mer peptide (results not shown). Acid elution and subsequent pool sequencing of the released peptides yielded the results and motif presented in Fig. 1A. Dominant residues (boxed values) as determined previously (12, 33) were seen at positions 2, 3, 4, 8, and 9. These results are comparable with the RT1-A\(^a\) motif results already published by Powis et al. (11) and reproduced in Fig. 1B. It can be noted that, in their results obtained from natural isolates, the residues which increased at positions 3 and 4 (Phe and Pro, respectively) were not seen as large enough increases to be considered anchor residues although both are present in the natural mitochondrial peptide ligand.

**RT1-A\(^a\) Peptide Motif from Recombinant and Natural Sources**—Having obtained successful results for RT1-A\(^a\), this method was used to refold around the TAP2-B-associated class I molecules, RT1-A\(^a\) and RT1-A\(^f\) (see below). Initial experiments with a 10-fold excess of random peptide:heavy chain failed to yield sufficient refolded complex. Only when the peptide ratio was increased to 100-fold did the complex refold sufficiently (see “Discussion”), with the peak area being 41% of the total area of the aggregate and complex (Fig. 7). With the higher ratio of peptide to heavy chain, the peptide motif for RT1-A\(^a\) was determined (Fig. 2A). RT1-A\(^a\) revealed a motif with strong preferences for hydrophobic and uncharged residues. Dominant residues appeared at positions 2, 3, 7, and 8 and, in
particular, showed a C-terminal preference for Tyr at position 9.

In addition, this RT1-Au motif was confirmed by performing an immunoprecipitation experiment from the C58 cell line using the RT1-Au specific monoclonal antibody, U9F4 (Fig. 2B). Results revealed dominant residues at positions 2, 3, 7, and 9, which were in agreement with the results from the in vitro refolding. Other positions in the motif showed some strong increases that were not present with the recombinant system. At position 4, there were strong increases for Asp and Arg, whereas an Ala was dominant at position 6.

RT1-A1c Peptide Motif from Recombinant and Natural Sources—As for RT1-Au, successful refolding with recombinant RT1-A1c was achieved only with a 100-fold excess of peptide over the heavy chain. The motif obtained from pool sequencing for recombinant RT1-A1c is shown in Fig. 3A. Dominant residues appeared at positions 2, 3, and 8 and with an almost singular preference for proline at position 2. Interestingly, there was an observed increase in tyrosine at position 8 while no obvious C-terminal anchor residue was observed at position 9, unlike for the two other class I heavy chains that we have used in this study. The motif derived from the rat splenocytes (Fig. 3B), revealed similar anchor residues to the recombinant system. The strong proline at position 2 is confirmed as is the preference for positive residues, particularly His and Arg at position 3. An increase for tyrosine at position 8 was observed, and in addition, a C-terminal (P9) anchor, leucine, was indicated.

DISCUSSION

In this study we have successfully used a random nonamer peptide library to determine the peptide binding motifs for RT1-Aa, RT1-Au, and RT1-A1c using a recombinant system. We describe a method to produce large amounts of rat MHC heavy and light chains. We have cloned, expressed, and purified truncated versions of three rat RT1-A class I heavy chains containing only the α1, α2, and α3 domains. Engineering a His-Tag at the C-terminal end of the heavy chains enabled a quick and simple purification strategy while leaving the protein denatured in 8 M urea and ready for refolding. Purification in the absence of reducing agents yielded both monomers and aggregates with interdisulfide bonds. The use of the Qiagen Ni-NTA matrix allowed for the enrichment of monomers from aggregates. Attempts to denature aggregates in the presence of
reducing agents and to refold them subsequently were unsuccessful (results not shown). The b₂m was purified by urea solubilization, followed by renaturation by dialysis. Ion exchange chromatography with a Q-Sepharose column successfully purified monomers from aggregates.

In recent years, large scale protein expression of MHC molecules in bacteria has mainly been directed toward crystal production for structure determination, when a binding peptide is already known (31, 32, 38–42). Here we present results showing that the same method can be adapted successfully to elucidate the binding motifs of rat RT1-A MHC molecules by using a random peptide library. Refolding with a fixed length random peptide library has a clear advantage over immunoprecipitation of MHC class I molecules expressed in mammalian cells. Unlike class II MHC molecules, peptides are usually locked into the groove of class I molecules at their N and C termini. In a natural system, peptides of different lengths can still bind to class I molecules (7, 14–16), and when eluted and subjected to pool sequencing, the C-terminal residues are often "diluted" away because of the different lengths of the population. This should not often occur with a fixed length random peptide library although it is possible for a nonamer library to bind with an end extending out of the groove. In addition, immunoprecipitation requires a rich source of class I and a specific antibody to extract a homogeneous population of the class I under study. This is often unavailable, especially with the poorly expressed non-classical (class Ib) molecules. The cloning scheme described here, coupled to refolding around random peptides, could also produce conformationally correct material for raising anti-class I antibodies. Raising antibodies against a complex with a specific peptide bound within the groove of a specific class I is also possible with this system. Injection of a single peptide-MHC complex, rather than a cell presenting the MHC with the peptide as was done by Porgador et al. (43), should produce more specific antibodies.

Only the monomer fractions for both subunits were used during the refolding experiments presented here. This enrichment produced reproducible refolding of between 35 and 45% of the starting material, when using both the specific 13-mer peptide and the random peptide library. This value is higher.

**Fig. 6.** Conformational experiments for RT1-A refolding with a specific 13-mer peptide. A, an ELISA assay was performed using the antibodies described. There is an increase in MAC 30 reactivity (Aα-specific) when peptide is present. The purified complex is both MAC 30 and C9 (anti-b₂m) positive. B, the complex was further analyzed by FACS analysis by coupling the complex to Ni-NTA silica via its His-Tag and probing the matrix with MAC 30 and a fluorescein isothiocyanate-coupled rabbit anti-rat-IgG secondary antibody.
than any previously reported for refolding with bacterial protein; 20% was the maximum yield reported by Reid et al. (32), who “pulsed” the refolding mixture with aliquots of heavy chain. We have yet to determine whether such pulsing could improve yields in our system. The purified complex was shown to contain both heavy and light chains, by SDS-PAGE and isoelectric focussing gel electrophoresis, and to have conformationally correct material, by ELISA and FACS. In this report, we have used Ni-NTA silica (Qiagen Inc.) for FACS analysis. The product, sold as a solid support for FPLC, HPLC, and microspin miniprep applications, has a bead size of 16–24 μm, which was small enough to be unhindered in the tubing of the FACSCalibur™ (Becton Dickinson). Binding via the His-Tag allows complete access of the antibodies to the native complex and produced an increase in fluorescence with the RT1-Aa specific antibody, MAC 30. Such material could provide a replacement for TAP-deficient cell lines such as RMA-S and T2, which are routinely used for providing “empty” class I molecules.

Pool sequencing results for acid-eluted random nonamer peptides from RT1-Aa, when compared with the motif reported by Powis et al. (11), show a comparable motif with identical amino acid preferences at positions 2, 8, and 9. In addition to these positions, our results suggest a strong preference for serine at position 1 and for phenylalanine and proline at positions 3 and 4, respectively. The latter two were both observed in the previous report but not as anchor residues. This discrepancy may be explained by reduced availability of such peptides in a natural system compared with the random library. Similarly, results for the TAP2-B-associated RT1-Au from both random peptide refolding and immunoprecipitation were comparable and revealed a general preference for aliphatic and aromatic residues at positions 2, 3, 6, and 7 and a strong preference for tyrosine at position 9 (even to the exclusion of Phe). As for RT1-Aa, there was also a preference for serine at position 1 in addition to threonine, alanine, and glutamic acid. The major differences between the two systems occur at positions 3 and 4, which once again may be due to a more stringent peptide availability in the natural system. Fig. 8 shows a comparison of the hydrophobic residues in the theoretical structures of RT1-Aa, RT1-Au, and RT1-A1c. The circled region clearly shows an increase in hydrophobic residues for RT1-Au in the β-sheets at the bottom of the groove. This provides a likely explanation for the hydrophobic residues observed at positions 6–8 of the immunoprecipitation motif (Fig. 2), which would fit into this area if placed in the groove.

In both the motifs for RT1-A1c, we find a strong preference for proline at position 2. This anchor residue has already been reported in other class I molecules such as H-2Ld (12), HLA-B7 (44), HLA-B*3501 (45), and HLA-B53 (46). It is of interest to note that the TAP/peptide translocation work of Neefjes et al. (46) found that a proline at position 2 or 3 of the peptide negatively influenced transport in both the rat TAP-A and TAP-B transporters (also observed with human TAP, Ref. 22). Our data do not necessarily contradict the TAP data since longer, Pro-containing peptides could be transported and amino-terminal trimming in the ER would make them suitable for binding to RT1-A1c.

Other interesting observations from the recombinant RT1-A1c are the significant increase in tyrosine at position 8 and surprisingly, no observed specific increase at the ninth position, whereas from the natural system (Fig. 3B), a leucine was observed at position 9. The C-terminal anchor residues for the other motifs reported, RT1-Aa and RT1-Au, were determined...
Fig. 8. Comparison of hydrophobic residues for RT1-A<sup>a</sup>, RT1-A<sup>a</sup>, and RT1-A<sup>c</sup>. Computer-generated models of the three RT1-A molecules used in this study. Sequences for residues 1–180 were submitted to the Swiss Model server (49–51). Returned theoretical structures were then edited to remove the α3 domains and displayed using the freeware program Rasmol. Hydrophobic residues (Ala, Leu, Val, Ile, Pro, Phe, Met, and Trp) are displayed as black areas. The circled area on each molecule shows the major differences for hydrophobicity.

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REFERENCES

1. Nefie, J. J., Momburg, F., and Hammerling, G. J. (1993) Science 261, 769–771
2. Shepherd, J. C., Schumacher, T. N. M., Ashton-Rickardt, P. G., Imaeda, S., Ploegh, H. L., Janeway, C. A., and Tonegawa, S. (1993) Cell 74, 577–584
3. Powis, S. J., Deverson, E. V., Coadwell, W. J., Ciruela, A., Huskisson, N. S., Smith, H., Butcher, G. W., and Howard, J. C. (1992) Nature 357, 211–215
4. Joly, E., Deverson, E. V., Coadwell, J. W., Günther, E., Howard, J. C., and Butcher, G. W. (1994) Immunochemistry 40, 45–53
5. Heemels, M. T., Schumacher, T. N. M., Wonigeit, K., and Ploegh, H. L. (1993) Science 260, 2059–2063
6. Momburg, F., Roelse, J., Howard, J. C., Butcher, G. W., Hammerling, G. J., and Nefie, J. J. (1994) Nature 367, 648–651
7. Heemels, M. T., and Ploegh, H. L. (1994) Immunity 1, 775–784
8. Köppmann, J. O., Post, M., Nefie, J. J., Hammerling, G. M., and Momburg, F. (1996) Eur. J. Immunol. 26, 1720–1728
9. Powis, S. J., Young, L. L., Barker, P. J., Richardson, L., Howard, J. C., and Butcher, G. W. (1993) Transplant. Proc. 25, 2752–2753
10. Reizis, B., Schild, H., Stevanovic, S., Mor, F., Rammensee, H. G., and Cohen, I. R. (1997) Immunochemistry 45, 275–279
11. Powis, S. J., Young, L. L., Joly, E., Barker, P. J., Richardson, L., Brandt, R. P., Melief, C. J., Howard, J. C., and Butcher, G. W. (1996) Immunity 4, 159–165
12. Falk, R., Rötzer, O., Stevanovic, S., Jung, G., and Rammensee, H. G. (1991) Nature 351, 290–296
13. Joly, E., Leong, L., Coadwell, W. J., Clarkson, C., and Butcher, G. W. (1996) J. Immunol. 157, 1551–1558
14. Gu, H. C., Jarzabek, T. S., Garret, T. P. J., Lane, W. S., Strominger, J. L., and Wiley, D. C. (1992) Nature 360, 364–366
15. Joyce, S., Kurushima, K., Kepecs, G., Angeletti, R. H., and Nathenson, S. G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 4145–4149
16. Urban, R. G., Chicz, R. M., Lane, W. S., Strominger, J. L., Rehm, A., Kenter, M. J. H., UytdeHaag, F. C. M., Ploegh, H., Uchanska-Ziegler, B., and Ziegler, A. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 1534–1538
17. Dooley, C. T., and Houghten, R. A. (1992) J. Med. Chem. 35, 1509–1517

alleles at RT1-A and TAP2 may reflect the related specificities of transporter and presenting molecule (4). However, since the permissible TAP-A transporter can also transport such peptides, the allelic associations may be based on other unknown factors.

It is also interesting to note that, whereas only a 10-fold ratio of peptide:heavy chain was required for refolding with RT1-A<sup>a</sup>, a 100-fold ratio was required for RT1-A<sup>A</sup> and RT1-A<sup>c</sup>. Since pool sequencing of the untreated random peptide library showed no significant enrichments for any amino acids at any position, indicating a random population, there is an implication that both of these molecules have a more stringent requirement for particular peptides, possibly as the groove requires specific types of residues at more positions than does RT1-A<sup>a</sup>. Alternatively, the increased preference for hydrophobic and aromatic residues in RT1-A<sup>a</sup> raises the possibility of reduced solubility for the preferred peptides and therefore reduced availability for refolding. But RT1-A<sup>c</sup> also requires a 100-fold ratio for efficient refolding, and this has charged residue preferences at position 3. In vivo, before peptides can bind to class I molecules for presentation, at least two points have been identified where they are processed: the proteasome where the peptides are produced and the TAP transporter which transports peptides into the endoplasmic reticulum lumen. Therefore, peptide presentation in natural systems is subject not only to what peptides can bind to the class I but also to how proteins are processed and what peptides are able to be transported into the ER lumen. The method we have used here is free from the cellular processing and transport and as such, measures only the specificity of the groove of the class I molecule itself. Comparisons of the motifs from natural and recombinant sources for both RT1-A<sup>a</sup> and RT1-A<sup>A</sup> do indeed show some differences, suggesting that the proteolytic and TAP transporter machinery may be imposing significant restrictions on the availability of peptides including positions other than the C-terminal anchor. If this were the case, then the method we describe here could prove extremely valuable for identifying such restrictions.
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18. Udaka, K., Wiesmüller, K.-H., Kienle, S., Jung, G., and Walden, P. (1995)  J. Exp. Med. 181, 2097–2108
19. Walden, P. (1996)  Curr. Opin. Immunol. 8, 68–74
20. Wiesmüller, K.-H., and Jung, G. (1995)  Biochem. Soc. Trans. 23, 678–681
21. Niedermann, G., Butz, S., Ehrenfeld, H. G., Grimm, R., Lucchiarri, M., Hochützky, H., Jung, G., Maier, B., and Eichmann, K. (1995)  Immunity 2, 289–299
22. Uebel, S., Kraas, W., Kienle, S., Wiesmüller, K.-H., Jung, G., and Tampe, R. (1997)  Proc. Natl. Acad. Sci. U. S. A. 94, 8876–8881
23. Uebel, S., Meyer, T. H., Kraas, W., Kienle, S., Jung, G., Wiesmüller, K.-H., and Tampe, R. (1995)  J. Biol. Chem. 270, 18512–18516
24. Laemmli, U. K. (1970)  Nature 227, 680–685
25. Fukumoto, T., McMaster, W. R., and Williams, A. F. (1982)  Eur. J. Immunol. 12, 237–243
26. Raghavan, M., Chen, M. Y., Gastinel, L. N., and Bjorkman, P. J. (1994)  Proc. Natl. Acad. Sci. U. S. A. 91, 2377–2381
27. Bhuyan, P. K., Young, L. L., Lindahl, K. F., and Butcher, G. W. (1997)  J. Immunol. 158, 1, 303–315
28. Metzger, J. W., Kempter, C., Wiesmüller, K.-H., and Jung, G. (1994)  Anal. Biochem. 219, 261–277
29. Wiesmüller, K.-H., Feierig, S., Fleckenstein, B., Kienle, S., Stoll, D., Herrmann, M., Jung, G. (1996) in Combinatorial Peptide and Nonpeptide Libraries-A Handbook for the Search of Lead Structures (Jung, G., ed), pp. 203–246, Verlag Chemie, Weinheim, Federal Republic of Germany
30. Garboczi, D. N., Hung, D. T., and Wiley, D. C. (1992)  Proc. Natl. Acad. Sci. U. S. A. 89, 3429–3433
31. Stevanovic, S., and Jung, G. (1993)  Anal. Biochem. 212, 212–220
32. Stephenson, S. P., Morley, R. C., and Butler, G. W. (1985)  J. Immunogenet. 12, 101–114
33. Stet, R. J. M., Zantema, A., Vanlaar, T., Dewaal, R. M. W., Vaessen, L. M. B., and Rozing, J. (1987)  Transplant. Proc. 19, 3004–3005
34. Hirel, P.-H., Schnitter, J. M., Dessen, P., Fayat, G., and Blanquet, S. (1989)  Proc. Natl. Acad. Sci. U. S. A. 86, 8247–8251
35. Madden, D. R. (1995)  Annu. Rev. Immunol. 13, 587–622
36. Silver, M. L., Parker, K. C., and Wiley, D. C. (1991)  Nature 350, 619–622
37. Zang, W. G., Young, A. C. M., Imarai, M., Nathenson, S. G., and Sacchettini, J. C. (1992)  Proc. Natl. Acad. Sci. U. S. A. 89, 8403–8407
38. Parker, K. C., Carreno, B. M., Sestak, L., Utz, U., Bidlison, W. E., and Coligan, J. E. (1992)  J. Biol. Chem. 267, 5431–5439
39. Collins, E. J., Garboczi, D. N., Karpusas, M. N., and Wiley, D. C. (1995)  Proc. Natl. Acad. Sci. U. S. A. 92, 1218–1221
40. Crowley, M. P., Reich, Z., Mavaddat, N., Altman, J. D., and Chien, Y. H. (1997)  J. Exp. Med. 185, 1223–1230
41. Porgador, A., Yewdell, J. W., Deng, Y., Bennink, J. R., and Germain, R. N. (1997)  Immunity 6, 715–726
42. Huczek, E. L., Bodnar, W. M., Benjamin, D., Sakaguchi, K., Zhu, N. Z., Shabanowitz, J., Henderson, R. A., Appella, E., Hunt, D. F., and Engelhard, V. H. (1993)  J. Immunol. 151, 2572–2587
43. Hill, A. V. S., Elvin, J., Willis, A. C., Aidos, M., Allsopp, C. E. M., Getch, F. M., Gao, X. M., Takiguchi, M., Greenwood, B. M., Townsend, A. R. M., McMichael, A. J., and Whittle, H. C. (1992)  Nature 360, 434–439
44. Neeffes, J., Gottfried, E., Roelse, J., Gromme, M., Obst, R., Hammerling, G. J., and Momburg, F. (1995)  Eur. J. Immunol. 25, 1133–1136
45. Matsumura, M., Saito, Y., Jackson, M. R., Song, E. S., and Peterson, P. A. (1992)  J. Biol. Chem. 267, 23599–23595
46. Davenport, M. P., Smith, K. J., Barouch, D., Reid, S. W., Bodnar, W. M., Willis, A. C., Hunt, D. F., and Hill, A. V. S. (1997)  J. Exp. Med. 185, 367–371
47. Peitsch, M. C. (1995)  Bio/Technology 13, 658–660
48. Peitsch, M. C., Wells, T. N. C., Stampf, D. R., and Sussman, J. L. (1995)  Trends Biochem. Sci. 20, 82–84
49. Peitsch, M. C. (1996)  Biochem. Soc. Trans. 24, 274–279
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