RT1-U: Identification of a Novel, Active, Class Ib Alloantigen of the Rat MHC


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RT1-U: Identification of a Novel, Active, Class Ib Alloantigen of the Rat MHC


In common with other mammalian species, the laboratory rat (Rattus norvegicus) expresses MHC class I molecules that have been categorized as either classical (class Ia) or nonclassical (class Ib). This distinction separates the class Ia molecules that play a conventional role in peptide Ag presentation to CD8 T cells from the others, whose function is unconventional or undefined. The class Ia genes are encoded by the RT1-A region of the rat MHC, while the RT1-C/E/M region encodes up to 60 other class I genes or gene fragments, a number of which are known to be expressed (or to be expressible). Here we report upon novel MHC class Ib genes of the rat that we have expression cloned using new monoclonal alloantibodies and which we term RT1-U. The products detected by these Abs were readily identifiable by two-dimensional analysis of immunoprecipitates and were shown to be distinct from the class Ia products. Cellular studies of these molecules indicate that they function efficiently as targets for cytotoxic killing by appropriately raised polyclonal alloreactive CTL populations. The sequences of these class Ib genes group together in phylogenetic analysis, suggesting a unique locus or family. The combined serological, CTL, and sequence data all indicate that these products are genetically polymorphic. The Journal of Immunology, 1999, 162: 743–752.

Maj or histocompatibility complex genes are typically classified into two groups: classical (class Ia genes) and nonclassical (class Ib genes). The class Ia genes have a broad tissue distribution and are abundantly expressed at the cell surface, i.e., on the order of $10^5$ molecules/resting lymphocyte. They perform a crucial role in immune surveillance by selectively binding intracellular peptides and presenting them at the cell surface to Ag-specific $\alpha \beta$ TCRs. They provoke strong allograft responses, the phenomenon that originally led to their discovery. Class Ia genes are highly polymorphic (e.g., >40 alleles at the human HLA-B locus, with many alleles also possessing several variant subtypes); each allele is capable of binding a different spectrum of peptides. By contrast, the properties of class Ib genes, e.g., H2-M3, Qa-1, and HLA-E (1), are more diverse, perhaps reflecting their adaptation to different, specialized functions. With exceptions such as Qa-2 (2), they are often expressed at a much lower density (some are not even expressed), and their tissue distributions range from rather limited (TL, HLA-G) to broad (Qa-2, H2-M3) (1). The genes also tend to monomorphism or oligomorphism rather than polymorphism.

To date, well over a hundred classical class I genes have been cloned and extensively studied in mammals as well as some other orders, such as birds and fishes. In comparison, nonclassical class I genes have received far less attention. Genes at three classical class I loci are expressed in humans (HLA-A, -B, and -C) and in mice (H2-K, -D, and -L). However, another 15–30 additional class I (Ib) loci are known to exist in humans, and >50 exist in the mouse, of which at least half are expressed (3).

In the rat the existence of at least 61 class I genes was indicated by a cosmid cloning analysis of the RT1<sup>el</sup> haplotype of the rat MHC (4). In contrast to mice and humans, the laboratory rat appears to possess either one or two functional classical class Ia genes, the number being haplotype dependent (5–7). These genes reside in the RT1-A region, at the centromeric end of the MHC on the short arm of rat chromosome 20 (8). The other cluster of class I genes, the class Ib genes, is located in the RT1-C/E/M region, which is separated from RT1-A by the class II (RT1-B/D), and class III regions. Analysis of recombinant and deletion mutant strains has mapped the majority of rat MHC class I genes to RT1-C/E/M (4, 9, 10). A limited number of rat MHC-encoded class Ib genes has been cloned and sequenced from different RT1 haplotypes. Some of these appear to correspond to genes well known from studies of mouse H2 (e.g., RT1-BM1, RT1-M3, and RT1-N) (11), while others have no close correspondence (e.g., the LW2 clone RT1-C<sup>2</sup>, RT1-E<sup>u</sup>, and Pa Ag) (12).

Our different laboratories have produced a number of MHC-specific monoclonal alloantibodies that recognize RT1-C/E/M-encoded alloantigens. We have made use of an expression cloning method (6) to identify the targets of some of these mAbs. Here we report the cloning of expressible gene sequences that we have designated RT1-U, derived from the RT1<sup>el</sup> and RT1<sup>h</sup> haplotypes.
Materials and Methods

Animals

Rats were either purchased from Harlan OLAC (Bicester, U.K.) or bred and housed under specific pathogen-free conditions at The Babraham Institute (Cambridge, U.K.), at the University of Oslo (Oslo, Norway), or at the Medizinische Hochschule (Hannover, Germany). MHC haplotypes of the rat strains used are detailed in Table III.

Monoclonal Abs

The mAbs used or derived in this study are listed in Table I. They were generally used as tissue culture supernatants. The spleen cell parent of the MAC 100 and MAC 101 hybridomas came from a (PVG-RT1a\textsuperscript{w} × BN)F\textsubscript{1} rat immunized with PVG.R19 splenocytes, restricting the allogeneic response to RT1-C\textsuperscript{c}. The spleen cell parent of the AAS hybridomas was a PVG.R8 rat immunized with bone marrow cells and lymphocytes (depleted of three sources: Con A blasts from two 4-mo-old female NEDH (RT1\textsuperscript{g}) rats and PC12 stimulated with 10% IFN-\textgamma) and PVG.R19 splenocytes, restricting the allogeneic response to RT1-C\textsuperscript{c}. The spleen cell parent of the AAS hybridomas was a PVG.R8 rat immunized with bone marrow cells and lymphocytes (depleted of three sources: Con A blasts from two 4-mo-old female NEDH (RT1\textsuperscript{g}) rats and PC12 stimulated with 10% IFN-\textgamma) and PVG.R19 splenocytes, restricting the allogeneic response to RT1-C\textsuperscript{c}. The spleen cell parent of the AAS hybridomas was a PVG.R8 rat immunized with bone marrow cells and lymphocytes (depleted of three sources: Con A blasts from two 4-mo-old female NEDH (RT1\textsuperscript{g}) rats and PC12 stimulated with 10% IFN-\textgamma) and PVG.R19 splenocytes, restricting the allogeneic response to RT1-C\textsuperscript{c}.

DNA cloning and sequencing

We adopted the protocol described by Joly et al. (6). Briefly, using the MicroFast Track and cDNA cycle kits (both from Invitrogen, San Diego, CA), CDNA was prepared from rats of the appropriate haplotype. For the c\textsubscript{c} series of clones, cDNA was prepared from about 25 million lymphocytes isolated from the spleen of a 6-mo-old female PVG.R19 rat (av1-avl-c, see Table III for genetic definition) followed by three independent PCRs. Twenty-four cc clones, i.e., eight clones from each PCR, were analyzed in transient transfections. After excluding clones on the basis of RT1-A serology, 15 clones were retained for further analyses. For the c\textsubscript{a} series of clones, cDNA was similarly prepared from about 25 million lymphocytes from a 6-mo-old male PVG-RT6(F344) rat (RT1-C\textsuperscript{c}) followed by two independent PCRs. Twenty c\textsubscript{a} clones, i.e., 10 from each PCR, were tested for RT1-A serology. Of these 20, 12 clones encoded RT1-A molecules and were excluded from subsequent analyses. To isolate clones of the RT1\textsuperscript{c} haplotype, cDNA was prepared from about 10 million cells of each of three sources: Con A blasts from two 4-mo-old female NEDH (RT1\textsuperscript{c}) rats (obtained from John Hutton, University of Cambridge, Cambridge, U.K.), the NEDH-derived PC12 adenral pheochromocytoma cell line (19), and PC12 stimulated with 10% IFN-\textgamma-supernatant. Each cDNA preparation was amplified by two independent PCRs. Of the 78 clones thus obtained, 41 were later eliminated on the basis of RT1-A serology.

Upstream and downstream primers used to amplify class I cDNA were 5'-TGC TGC TGG CGG CCC TGG-3' and 5'-GCT CTA GAG TCC-3', respectively. Amplified products were purified by agarose gel electrophoresis, ligated into the expression vector pCMU, and transformed into DH5\textalpha. Bacteria. As RT1-A\textsuperscript{c}, but not RT1-C\textsuperscript{c}, contains AvuI and KpnI restriction sites, plasmid DNA in some cases was enriched for RT1-C\textsuperscript{c} clones by restriction digestion with either of these enzymes before transformation into bacteria. Plasmid DNA prepared from bacterial colonies was transiently transfected into mammalian cells for screening by flow cytometry.

Plasmid DNA for sequencing and transfections was prepared in Epicurian coli XL1-Blue supercompetent cells (Stratagene, Cambridge, U.K.) in Luria-Bertoni medium overnight and purified with a midiprep or miniprep kit (Qiagen, Crawley, U.K.). Sequencing was performed on an ABI Prism 373A automated sequencer (Applied Biosystems, Warrington, U.K.) using AmpliTaq DNA polymerase and dsDNA. The upstream primer for sequencing was the same as that used for cloning (see above), while the downstream primer was 5'-GAA AGA ACA ATC AAG GGT CC-3', annealing to the pCMU vector. Each DNA clone was sequenced twice, in the 5' and 3' directions.

Sequence analysis

Sequences were edited using GCG software (University of Wisconsin Genetics Computer Group, Madison, WI); FASTA and BLAST were used for comparison with sequences in the EMBL database. Accession numbers (EMBL database) of novel or published sequences discussed in this paper are given in Table II.

Transfections and cell culture

Transient transfection of simian COS-7 kidney fibroblast cells and stable cotransfection of murine fibroblast L cells with the neomycin resistance gene were performed as previously described (6). The efficiency of transient transfections ranged from 22–40% for all clones. Transfected cells were immunohistochemically labeled for expression of class I products by flow cytometry. Transfected L cells were cultured under the selection of the antibiotic Genetin G-418 (Life Technologies, Paisley, U.K.). G-418-resistant L cells cotransfected with class II-bearing plasmids were separated from the cells expressing only the neomycin resistance gene by sterile cell sorting for MRC-OX18- or AAS2-positive cells on a FACStar Plus (Becton Dickinson, Erembodegem-Aalst, Belgium). Sorted cells expressing RT1-C\textsuperscript{c} molecules were plated out by limiting dilution, and 12 individual clones/DNA clone were picked for further characterization.

Induction by IFN-\gamma

Murine X63 cells (20) secreting mouse IFN-\gamma were cultured in RPMI and 10% FCS, and the culture supernatant was harvested just before the culture.

Table I. Locus and haplotype reactivities of anti-class I Abs used in this study

<table>
<thead>
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<th>Ab</th>
<th>Isotype</th>
<th>Specificity</th>
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<td>ART-02</td>
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Table II. Accession numbers (EMBL database) of novel or published sequences discussed in this paper

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<td>RT1-C\textsuperscript{c} clone cc9</td>
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reached confluence. L cell transfectants or lymph node cells were incubated for 48 h with 10% X63 supernatant in RPMI, 5% FCS, and 2.5 x 10^{-7} M 2-ME (Sigma, Poole, U.K.). The optimal concentration of X63 supernatant for use in culture was determined by titration of the supernatant for induction of class I visualized by flow cytometry.

Flow cytometry

Briefly, cells suspended in PFN (PBS, 2% FCS, and 0.05% sodium azide) were incubated with the primary Ab for 30 min on ice, then incubated under the same conditions with the secondary reagent. The cells were washed three times with PFN following each incubation, and their fluorescence was analyzed on a FACScan or a FACS Calibur (both from Becton Dickinson). When they were not analyzed on the same day, cells were fixed by resuspension in PFN and 1% formaldehyde. The primary Abs used are listed in Table I. Secondary reagents were FITC-conjugated rabbit anti- mouse Ig. FITC-conjugated rabbit anti-rat Ig (both from Dako, High Wycombe, U.K.), FITC-conjugated NORIG 7.16.2 (mouse anti-rat IgG2b (21), or MARG2a (mouse anti-rat IgG2a, Serotec, Kidlington, U.K.).

Immunoprecipitation of MHC class I molecules

PVG.R1 Con A blasts (25 x 10^6) or 10 x 10^6 L cell transfectants were labeled for 15 min at 37°C with 7.4 MBq of ^35 S-Trans label (ICN, Thame, U.K.), and the cell pellet was lysed in 1 ml of Nonidet P4-10 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 10 mM Tris (pH 7.4), and 1 mM PMSF) for 30 min on ice. Following a 5-min spin at 16,000 x g, the lysate was divided into aliquots and immunoprecipitated with 0.1 ml of tissue culture supernatant of the indicated mAbs and 30 µl of a mixture of protein A- and G-Sepharose (both from Sigma, Poole, U.K.). Immunoprecipitates were washed three times in 0.5 ml of Nonidet P-40 buffer and were analyzed by two-dimensional gel electrophoresis (22).

Cytotoxic T cell assay

PVG-R1 av1 (DA) rats were immunized i.p. and s.c. with 2 x 10^8 congenic PVG.R19 splenocytes. Three to eight weeks later, lymph node cells from immunized rats were restimulated for 4 days with irradiated PVG.R19 lymphocytes in RPMI (Life Technologies, Paisley, U.K.) supplemented with 5% FCS, 2.5 x 10^{-5} M 2-ME (Sigma), and 10% rat Con A supernatant. The resultant effectors were used in a standard CTL assay. Targets of Con A blasts or L cell transfectants (2-5 x 10^6) were labeled for 1 h at 37°C with 3 MBq of ^35 S-iodide (Amersham, Aylesbury, U.K.), then washed three times. Effectors and targets were plated out in triplicate in 200-µl volumes in microtiter plates in a range of E:T cell ratios, at 10^5 targets/well. In a cold target inhibition assay, cold targets were plated out at 10^4 targets/well. In both cases, the plates were spun at 800 rpm for 2 min and incubated at 37°C. After 4 h, 100 µl of supernatant was harvested from each well for counting on a gamma counter (Packard, Pangbourne, U.K.). Assays were performed three times.

Results

Novel mAbs

To enable specific detection of RT1-C products, two groups of mAbs were raised against alloantigens encoded by the RT1-C region of the c haplotype. These were MAC 100 and 101 (Cambridge), and the AAS series (Oslo), in both cases generated from the cross-reaction of this mAb with the RT1-A2c molecule (obtained from D. Lambracht-Washington, unpublished observation).

The AAS mAbs proved to be rather more cross-reactive, recognizing a number of RT1-A- and RT1-C-encoded products (Table III). They were generally similar in their patterns of reaction, although AAS5 was distinguished by much weaker cross-reaction against A1c and A2c, and AAS6 by its reaction on RT1-A^v. Together with cross-reactivity to RT1-C av1, we note the reactivity to L cells bearing cosmids from gene cluster 12 (viz., a33.1 and a4.2) of an RT1 av1 library (4).

Obtaining cDNA clones

Armed with these novel anti-RT1-C serological reagents, we embarked on cloning their target(s) from two haplotypes, RT1^v and RT1^v. We used a previously described method of PCR expression cloning that has been applied successfully to obtain classical class Ia MHC alleles from several rat strains (6, 23). The downstream primer used to amplify class I sequences is complementary to the 3' untranslated region downstream of and including the stop codon of the cloned RT1-A^v, while the sequence of the upstream primer is located 33 bases downstream of the translation start site of RT1-A^v, within the leader peptide-coding sequence. While these primers were originally selected for the cloning of classical class I molecules, we expected that they would also enable the cloning of nonclassical genes for three reasons. First, the upstream primer sequence in the leader exon is shared by most classical and nonclassical genes in mice and rats; in the few published nonclassical sequences mapping to the RT1-C region (e.g., LW2, 11/3R, both RT1^v haplotypes) (24, 25), this sequence spanned by the primer is identical with that in RT1-A^v. Second, the amplification of cDNA using this primer pair had previously been observed to generate clones (in addition to the class Ia alleles) whose expressed products, although positive with the MRC-OX18 Ab (which recognizes a broadly expressed rat class I epitope), were negative with anti-classical class I Abs (6). Third, at the downstream end there is impressive conservation of 320 base pairs of the 3' untranslated region of class I genes of mouse and rat (26).

Screening of clones

Transient transfection of pCMU ligation products from the three RT1^v sources (see Materials and Methods) yielded clones that could be distinguished from potential class Ia RT1-A^v by positive labeling with MRC-OX18 and a representative of the AAS series (AAS2), but not with the g-reactive anti-RT1-A mAbs F16.4.4, GN4/4, GN7/7, and YR2/69.

Similarly, RT-PCR cDNA clones in pCMU from PVG.R19 (av1-av1-c) cells (cc clones) and PVG (c-c-c) cells (c clones) were transfected transiently into COS-7 cells, and 22 clones showing null reactivity with anti-RT1-A (class Ia) mAbs (F16.4.4, HAM-2, MAC 30, YR5/12) were identified. Further serological testing of these 22 transfectants revealed three patterns of reactivity (data not shown). Firstly, most clones (the cc16 group) expressed epitopes not only for the broadly reactive anti-class I Ab MRC-OX18, but also for MAC 100, MAC 101, and the AAS series of Abs. Secondly, the transfectants of five additional clones (cc9, cc13, cc21, cc22, cc23) were null for the entire panel of Abs, while, thirdly, the transfectant of one clone (cc1) was positive for only MRC-OX18. On account of the novel characteristic of the g haplotype clones and the cc16 group of clones, defined both serologically and by sequence (see below), we have ascribed to them the new gene symbol RT1-U (27).

RT1-U^v and RT1-U^v sequences

The class Ib genes from PVG and PVG.R19 could be grouped according to their sequence similarities, which correlate with the three serological patterns described above. The majority of the
sequences fell into one group (cc16 group), most similar to the consensus sequence. This consensus nucleotide sequence, encoding a novel class Ib gene, is shown in Fig. 1, aligned with RT1-C\textsuperscript{g}, its closest relative, another novel expressed class Ib gene. The consensus sequence for RT1-C\textsuperscript{g} was arrived at by sequencing 10 clones from the RT1\textsuperscript{c} haplotype. The sequences of three clones were identical with the consensus, while the rest differed by fewer than 10 single nucleotide substitutions.

Of the 22 RT1-C\textsuperscript{c} clones sequenced, sequences of four of 16 RT1-U\textsuperscript{c} clones were identical with the consensus sequence. One of these, the cc16 clone, defining the canonical RT1-U\textsuperscript{c} sequence, was selected for further characterization by expression in L cells. Clone cc14, which differed by eight nucleotides, was also further analyzed in L cells in case it turned out to encode a distinct gene. PCR amplification of the cDNA, emphasize the importance of sequencing several clones.

The sequences of three clones (n.d. for AA5) were identical with the consensus, while the others were identical with the consensus except for 1–1.5 log\textsubscript{10} greater than 10 single nucleotide substitutions. Of the 22 RT1-C\textsuperscript{c} clones sequenced, sequences of four of 16 RT1-U\textsuperscript{c} clones were identical with the consensus sequence. One of these, the cc16 clone, defining the canonical RT1-U\textsuperscript{c} sequence,
RT1-C clone LW2 were the closest rat class I relatives in public databases, showing 92.2 and 91.7% identity with RT1-U, respectively. We therefore named the haplotype-derived sequence RT1-U g.

Additional clone sequences

The sequences of clones falling outside the cc16/RT1-U group were also analyzed. Clones cc22 and cc23 showed 99% nucleotide identity with the published clone 3.6 of the DA rat (RT1 av1 haplotype) (5), with identical deletions both in exon 5 and of the entire sixth exon. However, cc23 has additionally deleted the remaining 3’ stretch of exon 5, consequently shifting the position of the premature stop codon and its reading frame downstream of exon 6. Interestingly, the nucleotide and predicted peptide sequence of cc23 is identical with the partial cDNA sequence of clone RTS (26), and a cDNA clone has been isolated from the RT1 av1 haplotype whose partial sequence indicates an exon 4-exon 7 arrangement identical with that of cc23 (28). Two clones, 9.5 and 9.6, have also been identified in the RT1 f haplotype, which encode homologues of 3.6 (A.-F. Le Rolle, unpublished observation). The early termination codon in cc22 and cc23 suggests that these cDNAs may encode a soluble class I molecule, possibly that identified by Spencer and Fabre (29) in DA serum, although there is as yet no evidence that 3.6, cc22, or cc23 is successfully translated in vivo.

The unique expressed clone cc1, which was reactive only with MRC-OX18 Ab, did indeed have a unique sequence. Published sequences with closest identity are RT1-A2 o (88.8%), RT1-A2 f (88.1%), and LW2 (88.1%). However, since we have not obtained other clones with identical properties, we are not able to rule out the possibility that the clone is in some way artifactual. Finally, cc9 and cc13, the remaining null clones (identical in sequence), contain stretches of deletions in exons 2 and 5 that may preclude detectable class I surface expression.

Characterization of the expressed molecules in L cells

Representatives from each group of RT1-C clones previously shown to express in COS cells (clones cc16 and cc14) were stably transfected into mouse L cells. Unexpectedly, none of the L cell transfectants appeared to express any product unless they were first stimulated with IFN-γ for 2 days (see below). The fluorescence histograms for the cc16 L cell transfectants confirm the serological profile of the COS-7 transient transfectants and are depicted in Fig.
containing 10% IFN-γ Abs by flow cytometry. The transfectants had been cultured in medium shown). In general, RT1-U c and RT1-U g exhibit identical patterns also Table III. Finally, we record that although, in general, IFN-γ treatment was required to induce RT1-U expression on L cell transfectants, occasionally a culture would stain positively without it. We are uncertain whether this variability reflects the sensitivity of the staining procedure or an undefined aspect of the tissue culture conditions.

**Immunoprecipitation of AAS- and MAC 100/101-reactive class I molecules**

Using detergent lysates of Con A blasts derived from PVG.R1 (av1-c-c) lymph node cells, we tried to identify the class I products detected by the AAS series and MAC 100/101 mAbs by two-dimensional gel analysis. The results are displayed in Fig. 3A. A strong signal was obtained with AAS6, and weaker signals in the same position were obtained using MAC 100 and MAC 101. The polypeptide(s) detected by these Abs was distinct from the class Ia (RT1-A*) chains expressed by these cells, which migrated in a more acidic position (MAC 30). Comparing these precipitations with that using MRC-OX18, which has broad specificity for several class I molecules, signals corresponding to the AAS/MAC 100/MAC 101-reactive species and to RT1-A* could be identified, while other spots remain unassigned (MRC-OX18 panel). Finally, immunoprecipitations of L cells transfected with RT1-U* produced a spot in the same position as that seen in the lymphoblasts, while control L cells transfected only with the neomycin resistance gene lacked this spot (Fig. 3B), confirming that the species in this gel position is determined by the cloned cDNA for RT1-U*.

**Sequence comparison of RT1-U1*, RT1-U2*, and related sequences**

We have proposed the names RT1-U1* and RT1-U2* for our c and g haplotype molecules described here (27). We assign them to the same family (RT1-U) because of the degree of their sequence identity (discussed above). We have, however, grouped them into different subfamilies (RT1-U1 and RT1-U2) because of their differing sequences in exon 5 (Fig. 4, A and B). Furthermore, other published sequences segregate with RT1-U1*, falling into the U1 group: firstly, the A-1 partial sequence (also RT1* haplotype) (30) is identical throughout to our RT1-U1* sequence (Fig. 1); and secondly, clone 109 (RT1*n haplotype) (31) is sufficiently similar to RT1-U1* that it may be the allelic RT1-U1*n form (Fig. 4A). If so, this is, to our knowledge, the first definition of an expressed allelic pair of class Ib genes in the rat.

**Presentation of RT1-U as an antigenic target to T cells**

We next asked whether our novel RT1-U molecules were capable of being recognized by T cells. To generate CTL specific for RT1-C*, we used CTLs of a specificity originally described by Stephenson and Butcher (33). We primed PVG-RT1 av1 (DA) rats with PVG.R19 splenocytes and then challenged them in vitro with PVG.R19 LN, eliminating the possibility of any dominant anti-RT1-A response. The resultant effectors displayed high levels of killing of RT1-U2* transfectants, although, noticeably, not as high as those for PVG.R19 Con A blasts (Fig. 5A). Allowing for differences in cell type and levels of expression of Ag, we conclude that there may be molecules in addition to RT1-U2* encoded in the RT1-C* region in PVG.R19 cells that are recognized by the CTL. Consistent with this interpretation, RT1-U2* transfectants as cold targets could not inhibit the lysis of PVG.R19 hot targets as efficiently as the homologous cold targets did (data not shown). IFN-γ has an impact on the killing of L cell transfectants; this effect will be discussed below.
C1), X90375 (RT1-A1 n), X90376 (RT1-A2 n), Y08532 (RT1-A g), X82108 (RT1-A u), X16979 (RT-BM1), L16012 (RT1-O), and L81134 (RT1-S1).

specific for RT1-U2 c is not able to recognize RT1-U1 g (Fig. 5A). Similarly, RT1-U1 g transfec-
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tc targets, we believe that the observed killing of RT1-U2 targets reflects the direct recognition of RT1-U2 as a discrete allogeneic target. Clearly, we have demonstrated the potential of RT1-U as an Ag-presenting molecule in vivo. Issues that are of interest to address in the future are the nature of the peptides that can bind and that are bound to these molecules and the physiological relevance of the Ags presented.

**FUNCTIONAL POLYMORPHIC DIFFERENCES BETWEEN RT1-U1<sup>g</sup> AND RT1-U2<sup>c</sup>**

The small number of nucleotide substitutions between RT1-U1<sup>g</sup> and RT1-U2<sup>c</sup> can apparently account for their functional polymorphism, i.e., their differential binding of MAC 101 mAb and their differential recognition by allogeneic T cells (Fig. 5). Of these nine base substitutions, five translate into coding changes: residue 69 (E–G) in the α1 domain (U2<sup>c</sup>-U1<sup>g</sup>) and residues 103 (L–V), 116 (S–H), 119 (N–D), and 163 (T–K) in the α2 domain. These coding substitutions involve a change in the size and significant changes in the charge (except residue 103) of the residue side chains, with residues 163 and 116 having the potential to influence the type of peptide that is preferentially bound by RT1-U. According to the structure of class Ia molecules determined by x-ray crystallography, residue 163 contributes to the P1 binding pocket (35). Residue 116 sits on the floor of the carboxyl-terminal binding pocket, whose other key peptide binding residues are conserved between U1<sup>c</sup> and U2<sup>c</sup> (F74 and N77), the two aromatic groups in U1<sup>g</sup> (H116 and F74) suggesting a preference for a small hydrophobic C-terminal residue. Last, 119 is implicated in interdomain hydrogen bonding with β<sub>m</sub> (35), while 69 (usually G or E in RT1-A molecules) may be accessible to TCR contact and hence may affect alloreactivity.

In general, other studies (reviewed by Madden (36)) have shown that TCR recognition of MHC will be affected by the primary sequence of an MHC-bound peptide, its solvent-exposed side-chain residues, as well as the conformation of the MHC polypeptide(s), which, in turn, may be influenced by the nature and packing of the peptide bound. In particular, the highly variable middle region of the extended MHC-bound peptide displaying solvent-exposed residues, contributes significantly to the antigenic identity of the MHC peptide complex. Furthermore, discriminating allo-reactive CTL can be obtained for very small differences in structure and even hidden side chains of bound peptide (37). The absence of three-dimensional structural information and information on peptides bound by RT1-U<sup>c</sup>, however, reduces the feasibility of making detailed predictions of the effects of these polymorphic differences on conformational changes in the MHC molecule.

**DISCUSSION**

The class Iб genes that we have cloned from the RT1<sup>g</sup> and RT1<sup>g</sup> haplotypes of the rat can be clustered by their degree of sequence similarity in a group that we have named RT1-U, distinct from other rat class I loci. In comparing sequences across exons 4–8, rat
 class Ia genes (e.g., RT1-A\textsuperscript{a}, RT1-A1\textsuperscript{c}, RT1-A2\textsuperscript{c}, RT1-A\textsuperscript{g}, RT1-A1\textsuperscript{a}, RT1-A2\textsuperscript{a}, and RT1-A\textsuperscript{A}) and some rat class Ib genes (clone 149, DA clone 3.6, RT1-C2, and RT1-E\textsuperscript{a}) are more similar among themselves than they are to some other rat class Ib genes (RT1-C1, RT-BM1, RT1-O, and RT1-S1; Fig. 4B).

In the \(\alpha\)1 and \(\alpha\)2 domains, an inspection of codon usage of nucleotide G or C at the third position reveals the frequency in RT1-U\textsuperscript{U1g} and RT1-U\textsuperscript{U2c} at asparagine 86 (NQS), as found in all rat, mouse (including Qa and Tla), and human class I sequences, and those of RT1-U\textsuperscript{U1g} and RT1-U\textsuperscript{U2c} are also found in RT1-A\textsuperscript{a}, RT1-A\textsuperscript{g}, RT1-A\textsuperscript{1A}, RT1-A\textsuperscript{2A}, and RT1-A\textsuperscript{A}). The four cysteine residues in the \(\alpha\)3 domain tend to be relatively constant across all rat class Ia and class Ib genes, ranging from 63.4–68.5% (with both RT1-U genes at 68%). Based on the hypothesis that the earlier the presumed evolutionary divergence of a class Ib gene from the ancestral class I gene, the less frequent the usage of G or C at the third position in the \(\alpha\)3 domain, the inspection of codon usage of nucleotide G or C at the third position reveals the frequency in all rat class Ia genes (RT1-A\textsuperscript{a}, 84.6%; RT1-A\textsuperscript{A}, 85.7%; RT1-A\textsuperscript{1A}, 82.9%; RT1-A\textsuperscript{1A}, 83.5%; RT1-A\textsuperscript{2A}, 81.8%; RT1-A\textsuperscript{A}, 83%; RT1-A\textsuperscript{2A}, 83.5%). The frequencies in the \(\alpha\)3 domain tend to be relatively constant across all rat class Ia genes.

The MHC class I \(\alpha\) domain, containing principal sites for binding \(\beta\)\(_m\) and CD8, remains the most highly conserved domain across class Ia and Ib molecules (see above), and class Ib molecules such as H2 M3 and Qa-2 have been found to be associated with \(\beta\)\(_m\). Certain HLA-A2 \(\alpha\)3 domain residues that have been implicated in forming polar (H192, E232, R234, P235, A236, G237, W244) or hydrophobic (R202, W204, V231, R234, P235, G237, D238) interactions with \(\beta\)\(_m\) (35) are all conserved in RT1-U\textsuperscript{U1g} and RT1-U\textsuperscript{U2c}, with the prediction that these molecules are capable of binding \(\beta\)\(_m\) in vivo. What about the likelihood of binding CD8? RT1-U\textsuperscript{U1g} and RT1-U\textsuperscript{U2c} may, like TL (40) rather than Qa-2 (41), be able to ligate CD8, because, with the exception of two conservative changes at Q224L and T228M, the residues crucial for the association of class I with CD8 (42, 43) have all been conserved (\(\alpha\)2 residues Q115, D122, E128, A245, and \(\alpha\)3 residues 223–9 (DQTQDTE)).

Could these class Ib Ags behave as T cell targets in vivo? Mouse TL (44) and Qa-1\textsuperscript{b} (45) behave as weak transplantation Ags and are capable of eliciting alloreactive CTL responses. Likewise, our novel RT1-U\textsuperscript{U1g} molecule is capable of being recognized as an allogeneic T cell target (Fig. 5). Strong anti-RT1-C\textsuperscript{c} and anti-RT1-C\textsuperscript{av1} CTL responses have been generated previously in RT1-C\textsuperscript{mismatched (but RT1-A- and class II-matched) congenic rat strains following in vivo priming; skin grafts exchanged between such RT1-C-mismatched rat strains are rejected relatively slowly over 14–30 days (33, 46). While we had difficulty in serological detection of RT1-U\textsuperscript{U2c} on untreated L cell transfectants (see above), it was not difficult to elicit CTL killing of the same transfectants without prior treatment with IFN-\(\gamma\), although IFN-\(\gamma\)-treated transfectants were killed somewhat better than resting ones. Since Eisen and co-workers have estimated that a CTL may need to ligate only a single MHC-peptide complex per target cell for cytolytic activation (47), this result is not necessarily surprising. Apart from increasing the level of RT1-U\textsuperscript{U2c} on untreated L cell transfectants (see above), it was not difficult to elicit CTL killing of the same transfectants without prior treatment with IFN-\(\gamma\), although IFN-\(\gamma\)-treated transfectants were killed somewhat better than resting ones. Since Eisen and co-workers have estimated that a CTL may need to ligate only a single MHC-peptide complex per target cell for cytolytic activation (47), this result is not necessarily surprising.

Apart from increasing the level of RT1-U\textsuperscript{U2c} on the L cell transfectants to serologically detectable levels, IFN-\(\gamma\)-treated and -untreated L\textsuperscript{U2c} cells were equivalent as cold inhibitors of the killing of untreated L\textsuperscript{U2c} targets, this was not so for IFN-\(\gamma\)-treated L\textsuperscript{U2c} targets. In this case, the IFN-\(\gamma\)-treated inhibitors were markedly superior. The mechanism of this effect is unknown; many of the components of the MHC class I Ag presentation machinery are sensitive to IFN-\(\gamma\), e.g., TAP, LMP2, and LMP7. If the CTLs used are sensitive to peptides bound in the U\textsuperscript{2c} peptide binding groove, then perhaps qualitative changes in peptide presentation induced by IFN-\(\gamma\) are responsible (48, 49).
What is the functional significance of RT1-U genes? On the premise that the presentation of peptides to cells of the immune system is performed by a limited number (i.e., two or three) of highly polymorphic class Ia genes, Klein and O’Huigin (50) postulate that class Ib genes serve the immune system as a reservoir of donor genes enabling diversification of class Ia genes through mechanisms such as gene duplication, conversion, and rearrangement, thereby allowing a population to adapt to changing environments. Conversely, a contemporary class Ib gene product may once have been one of the few primary Ag-presenting molecules, but now it no longer functions satisfactorily in the context of changed environmental pathogens and has been replaced by a novel molecule: as a discarded remnant of MHC evolution, it resides in the genome as a pseudogene (or underexpressed gene) with no apparent function.

While this may be true for some class Ib genes, there is strong and increasing evidence that several class Ib gene products bind and present special ligands and are extremely valuable in immune responses to bacteria, viruses and to stress proteins. Examples include murine H2-M3 (37), Qa-1 (51), Qa-2 (52), HLA-E (53), HLA-G (54), and MICA/MICB (55). Since they present Ag from cellular or pathogenic sources that may not be efficiently presented by class Ia molecules or else report on their cell’s infection or other distress, the specialized properties unique to several class Ib molecules serve their host well by complementing conventional peptide presentation by class Ia molecules. The rat is unusual in having only one or two class Ia molecules per haplotype (7, 23), while most mammals investigated have two or three. It may compensate for a less than optimal number by having an additional, small set of genes that it can use rather like class Ia genes (for example, presentation of alloantigen to T cells as seen in Fig. 5). The codon usage of G and C, as discussed above, also suggests that RT1-U is very class Ia-like, cf., all known mouse class Ib genes. Such class Ia-like nonclassical genes might be appropriately described as neo-classical to distinguish them within the very diverse collection of class Ib genes.

To date, the crystal structures of two MHC-encoded class Ib molecules have been reported: H2-M3 (37) and HLA-E (56). In general, their overall structures resemble those of class Ia molecules. The peptide binding region responsible for the binding of antigenic peptide by class Ia molecules comprises the α1 and α2 domains that are encoded by exons 2 and 3, respectively. The majority of interallelic nucleotide substitutions occur in these two domains, generating population diversity in the ability to bind various peptides, with the α3, transmembrane, and cytoplasmic domains remaining highly invariant. RT1-U shares with class Ia genes some highly conserved features that are thought to facilitate the accommodation of an extended peptide in the peptide binding groove. Canonical tyrosines at positions 7, 59, 59, and 171, involved in a network of hydrogen bonding to the N-terminus of the peptide ligand, are conserved in RT1-U, as are Y84, T143, K146, and W147 involved in hydrogen bonding to the ligand’s C-terminal residue (36).

Residues lining the P1 pocket (which accommodates the side chain of the ligand’s N-terminal residue) important in ligand binding, either follow the canonical residues of rat class Ia molecules or use alternative residues, which are also found in some rat class Ia members (S99, S167). Of the single polymorphism between RT1-U1 and RT1-U2 in this region, i.e., K163T, T163 (RT1-U2) also occurs in some rat class Ia molecules, but K163 (RT1-U1) is not used by any of the known rat class Ia molecules. The P2 pocket of RT1-U largely follows the canonical pattern of rat class Ia molecules, except for S99 (typically Y or F), which is found only in RT1-A1. Of the residues in the C/F pocket, T80, Y84, T123, and K146 follow those of all RT1-A alleles, which are absolutely invariant, and W147, which is invariant among mouse and human class Ia molecules. Of residues 74, 77, 81, 97, 114, 116, 141, and 150, which are polymorphic among rat class Ia molecules, RT1-U uses residues that occur in RT1-A molecules of at least one rat strain. Some of these variant residues (81, 141, 143, 147, and 150) are found in haplotypes naturally associated with Taps, while others (74, 77, 114, and 116) are found in haplotypes naturally associated with Taps (24). Although T132 and T134, which were reported to be crucial for interaction of class I with other components of the class I assembly complex (57, 58), are conserved in RT1-U, it is not clear whether the peptide binding preferences of RT1-U are differentially associated with either allele of rat TAP.

Residues forming the α1 and α2 domains of RT1-U diverge from the consensus rat class Ia in those stretches that are highly polymorphic (62–77 and 141–157) or moderately polymorphic (21–50, 78–116, and 158–186) between individual RT1-A molecules. Apart from K41, R57, E76, and 959 unique to RT1-U, the variant residues are also used by various RT1-A members. Of these unique residues, R57 and E76 may have an impact on MHC-TCR contact, as they are located on the α1 helix’s surface that points toward the TCR. A strikingly unusual feature occurs in the stretches that are invariant across all RT1-A molecules (residues 10–21 and 117–140), where six substitutions are found in RT1-U: M12, G14, K19, N119 in RT1-U2, V125, and T138. While perhaps not directly involved in binding the peptide ligand, changes in the first stretch of residues 10–21 carry the potential of altering the preferences of the P2 pocket, residue 119 is involved in interdomain H-bonding with β3m, and 125 and 138 are located near the interface of the CD8 binding site as discussed above, but may alternatively be involved in contact with molecules with which MHC class I interacts, such as TAP, tapasin, calreticulin, calnexin, NK receptors, or other unknown proteins.

What might be the tissue distribution pattern of RT1-U apart from peripheral lymphocytes? Northern blotting of various tissues may yield further answers. Once known, it would be of interest to determine the peptides naturally bound by class Ib genes such as RT1-U and, if they are expressed in the thymus, the nature of any role they play in T or NK cell maturation.

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