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Molecular Basis for Recognition of an Arthritic Peptide and a Foreign Epitope on Distinct MHC Molecules by a Single TCR

Devraj Basu,* Stephen Horvath,* Isao Matsumoto, † Daved H. Fremont,* and Paul M. Allen2*

KRN TCR transgenic T cells recognize two self-MHC molecules: a foreign peptide, bovine RNase 42–56, on I-Ak and an autoantigen, glucose-6-phosphate isomerase 282–294, on I-Ag7. Because the latter recognition event initiates a disease closely resembling human rheumatoid arthritis, we investigated the structural basis of this pathogenic TCR’s dual specificity. While peptide recognition is altered to a minor degree between the MHC molecules, we show that the receptor’s cross-reactivity critically depends upon a TCR contact residue completely conserved in the foreign and self peptides. Further, the altered recognition of peptide derives from discrete differences on the MHC recognition surfaces and not the disparate binding grooves. This work provides a detailed structural comparison of an autoreactive TCR’s interactions with naturally occurring peptides on distinct MHC molecules. The capacity to interact with multiple self-MHCs in this manner increases the number of potentially pathogenic self-interactions available to a T cell. The Journal of Immunology, 2000, 164: 5788–5796.

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3Abbreviations used in this paper: RA, rheumatoid arthritis; GPI, glucose-6-phosphate isomerase; RN, bovine RNase; tg, transgenic; NOD, nonobese diabetic; CHO, Chinese hamster ovary; HEL, hen egg lysozyme, g7-M, g7-mimic.

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heumatoid arthritis (RA) is a highly prevalent and debilitating autoimmune disease that is characterized by a relapsing symmetric synovitis of the peripheral joints (1). T cells have been implicated in RA pathogenesis both by their presence in joint lesions (2) and the association of particular MHC class II molecules with disease risk and progression (3–5). While these mice express I-Ak as their exclusive MHC class II molecule, it was originally presumed to be the only MHC ligand that this TCR could recognize in a self-restricted fashion. However, KRN tg T cells cause disease in K/B N mice, which express I-Ak and I-Ag7 but not I-Ak; therefore they must interact productively with another self-MHC class II molecule. Accordingly, it has been shown that KRN tg T cells from a C57BL/6 (H-2b) background (K/B mice) proliferate in response to splenic APCs that express I-Ag7. Crossing K/B mice to NOD mice (H-2g7) produces the arthritic F1 K/B × N offspring in which KRN T cells partially escape tolerance induction and exist in a state of systemic self-reactivity (6).

An autotransplant of target KRN T cells was discovered through inquiry into the pathogenic role of B cells in this model (8). The development of arthritis was shown to depend upon a B cell function that required I-Ag7-restricted KRN T cell help. This function proved to be the production of IgG autoantibodies, which alone will transfer arthritis from diseased K/B × N mice to normal B6 mice. A subsequent study has identified the self-protein targeted by autoantibodies in K/B × N mice as glucose-6-phosphate isomerase (GPI) (9). GPI is a ubiquitously distributed cytoplasmic enzyme that catalyzes the second step of glycolysis and is also found at low levels in serum. Remarkably, GPI was found also to stimulate KRN T cells when processed and presented by NOD APCs, thus identifying it as a T cell autoantigen as well.

Here KRN TCR’s interaction with GPI/I-Ag7 can be treated as self-recognition and not alloreactivity because KRN T cells encounter I-Ag7 as a self-restricted ligand in the context of a K/B × N mouse; thus, the common distinction between allo- and self-recognition proves to be an artificial one in this setting. In an H-2k/g7 mouse, KRN T cells then have a peculiar kind of dual specificity: they can recognize a foreign peptide on one self-restricted MHC molecule (RN42–56/I-Ak) or initiate autoimmune arthritis by seeing an autoantigen on a distinct self-MHC ligand (GPI/I-Ag7). The pathogenic importance of the latter recognition has led us to investigate the structural basis of this cross-reactivity. While many structural studies of multiple peptide recognition on a single self-MHC molecule exist (10, 11), precedents for dual MHC recognition by one TCR involve alloreactivity (12) or xenoreactivity (13).

In the present study, we identify the epitope targeted by KRN T cells in the GPI protein and assess the contact requirements for its recognition on I-Ag7 by this TCR. By comparing peptide specificity on I-Ak and a mutant I-Ak containing five residues from the I-Ag7 contact surface, we delineate how a single TCR can respond...
to foreign peptides and an arthritic self-peptide on distinct self-MHC ligands.

Materials and Methods

Mice

NOD/LtJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). K/B mice (a gift of D. Mathis and C. Benoist) were rederived and maintained in our colony by breeding to C57BL/6. The KRN TCR transgene was screened using PE-conjugated anti-mouse CD4 and FITC-conjugated anti-mouse Vβ 6 (PharMingen, San Diego, CA) dual FACS staining of peripheral blood. K/B.AKR mice were derived by breeding to the companion B6.AKR (H-2k) background for two generations and screening by FACS for the absence of Kβ expression using a biotinylated anti-mouse H-2Kβ Ab (PharMingen) plus PE-streptavidin (Caltag, San Francisco, CA). All K/B and K/B.AKR mice used were heterozygous for the TCR transgene.

Peptides

Peptides were synthesized using F-moc chemistry on a Rainin Symphony Multiplex peptide synthesizer (Rainin Instruments, Emeryville, CA) containing 10% FCS, 5 μg/ml gentamicin. The rest of the assays were performed in RPMI 1640 medium containing 10% FCS, 5 × 10–5 M 2-ME, 1 mM Glutamax, and 50 μg/ml gentamicin. NOD splenic APCs received 2000 rad gamma-irradiation before use. Before use, CHO-g7 and C3-AΔ5 APCs were incubated in the presence of 75 μg/ml mitomycin C (Sigma, St. Louis, MO) at 37°C for 2 h and then washed three times in HBSS. Proliferation was measured in 96-well tissue culture plates (Costar), which were pulsed at 48 h with 0.4 μCi [3H]thymidine and harvested 18–24 h later as described (15). Proliferation is expressed as counts incorporated (mean of duplicate wells).

I-Aβ mutagenesis

The I-Aαα- and I-Aαβ-chains were changed in their expression vectors, pcDNA3.1neo-i-Aα and pcDNA3.1neo-i-Aβ (a gift of E. R. Unanue), using PCR. The following mutagenesis were made with the indicated nonoverlapping primers: 5′-GTCAGAAAGGACCTCGAGGCCCTCCTCCAGC-3′ (coding), 5′-CTTCTAGTTGACCAACCTGGAAGC-3′ (non-coding); 5′-CGTGGTCGACAGAACAATCAGGAA-3′ (coding), 5′-GCTGGTGCCAGCGCTCGCCG-3′ (non-coding); 5′-GGCGTGCAGACAACATCAGGAA-3′ (coding), 5′-GCTGGTGCCAGCGCTCGCCG-3′ (non-coding); 5′-CTTCCGTCTCTGTGATTTGGTCTGG-3′ (non-coding). Plasmid sequences were amplified using Vent polymerase (New England Biolabs, Beverly, MA) as per manufacturer's instructions and then gel purified. Plasmids were circularized by ligation. A fifth mutation, 665[T→A], was made using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA) with the primers, 5′-GTCGAGAAGGACCTCGAGCCCTCCTCAGC-3′. The complementing vectors were named pcDNA3.1neo-i-Aα/153/A65 and pcDNA3.1neo-i-Aβ/153/B78/884/A187. Both mutant chains were fully sequenced.

Generation of I-Aβ expression constructs

The I-Aαα- and I-Aαβ-chain coding sequences were extracted from the vectors, pCEP4-Aα and BCMGSNeo-Aβ (a gift of O. Kanagawa), using PCR. Sequences were amplified using cloned Pfu polymerase (Stratagene) and the following primers, which added HindIII and BamHI sites at the respective 5′ and 3′ ends: 5′-ATATAAGGTATGTCGACGACAGCGCCTCT-3′ (5′ I-Aαα); 5′-ATATAGATGATCTACTAAGGGCTGGTGTACG-3′ (5′ I-Aαβ); 5′-ATATAGATGATCTACTAAGGGCTGGTGTACG-3′ (5′ I-Aαβ); 5′-ATATAGATGATCTACTAAGGGCTGGTGTACG-3′ (5′ I-Aαβ); 5′-ATATAGATGATCTACTAAGGGCTGGTGTACG-3′ (5′ I-Aαβ); 5′-ATATAGATGATCTACTAAGGGCTGGTGTACG-3′ (5′ I-Aαβ); 5′-ATATAGATGATCTACTAAGGGCTGGTGTACG-3′ (5′ I-Aαβ); 5′-ATATAGATGATCTACTAAGGGCTGGTGTACG-3′ (5′ I-Aαβ). PCR products of the α- and β-chains were digested and directionally cloned into the respective pcDNA3.1neo+ and pcDNA3.1neo+ expression vectors (Invitrogen, Carlsbad, CA) to generate the plasmids pcDNA3.1neo-i-Aαα and pcDNA3.1neo-i-Aαβ. Both chains were fully sequenced.

Generation of CHO-g7 and C3-AΔ5 cell lines

Chinese hamster ovary (CHO) cells were cotransfected with the pcDNA3.1neo-i-Aα and pcDNA3.1neo-i-Aβ vectors using the CellFEC-TIN liposomal reagent (Life Technologies, Gaithersburg, MD) as per manufacturer's instructions. Dual transfected cells were selected and maintained in 0.5 mg/ml G418 (Calbiochem, San Diego, CA) plus 0.5 mg/ml Zeocin (Invitrogen). M12.C3 cells were electroporated with pcDNA3.1neo-i-Aα/153/A65 and pcDNA3.1neo-i-Aβ/153/B78/A84/A187 plasmids. Electroporation was performed at 250V, 960 μF with a 0.4-cm gap cuvette (Bio-Rad, Hercules, CA) containing 10 μg of each plasmid, 5 × 106 cells, and 0.05 M HEPES in a volume of 300 μl medium. After 24 h of growth in bulk, cells were selected in 1 mg/ml G418 and were divided by limiting dilution across four 96-well tissue culture plates (Costar, Corning, NY). Drug-resistant CHO-g7 and C3-AΔ5 cells were stained for surface MHC expression using biotin-10,3.6.3 (anti-mouse I-Aβ/β; PharMingen) or biotin-11.5.2 (anti-mouse I-Aγ/α; PharMingen) plus PE-streptavidin; they then were sorted on a FACSVantage instrument (Becton Dickinson, Mountain View, CA).

I-Aβ binding assay

Relative I-Aβ binding strength was determined in an assay using substituted test peptides to compete against an 125I-labeled standard peptide (sequence YEDYGILQNSR) for binding of soluble I-Aβ, as previously detailed (14). The concentrations of test peptides required to inhibit 50% binding of the labeled standard were measured. The concentration of substituted test peptide relative to the concentration of RN92–56 required for 50% inhibition was calculated as the RIC–1 (relative inhibition) = concentration of test peptide/concentration of RN92–56.

Proliferation assays

Assays using CHO-g7 cells as APCs were performed in MEMa medium supplemented with 5% FCS, 1 mM Glutamax (Life Technologies), and 50 μg/ml gentamicin. The model was based on the hen egg lysozyme (HEL)50–62/I-Aα crystal structure (16) and involved replacement of 12 side chains. Each of the side chains were positioned individually into the lowest energy rotamer. Energy minimization studies, performed using the Discover module software (Micron Separations), indicated that the model was energetically favorable.

Results

A defined epitope in the GPI protein stimulates KRN T cells on I-Aβ

To better characterize the self-recognition event that initiates disease in K/B × N mice, we defined the precise amino acid sequence in the GPI protein that interacts with KRN T cells. GPI is a 558-aa protein (17) that was divided by peptide synthesis into 24 contiguous peptides, each overlapping with adjacent peptides by 13 aa at their termini. When presented by NOD APCs to KRN T cells from tg mice, only one peptide, GPI278–313, induced significant proliferation above background (Fig. 1A). Based on the failure of the two adjacent peptides to stimulate, the first and last 13 aa of GPI278–313 were ruled out as containing the complete T cell epitope. Among a series of other peptides tested within this sequence, a 13-aa sequence axis near the amino terminus, GPI282–294 (Fig. 1B) was found to stimulate sufficient TCR recognition of GPI294–313, a peptide spanning from the latter half of GPI282–294 to the carboxyl terminus of GPI278–313 (Fig. 1B) was assayed. This peptide, GPI287–313, failed to stimulate (Fig. 1C). Thus the self-GPI protein contains a single epitope encompassed by GPI282–294 that is recognized by KRN T cells when bound to I-Aβ.

Four amino acids of RN42–56 bound to I-Aβ can contact a TCR

Comparing recognition of RN42–56/I-Aβ and GPI282–294/I-Aβ requires knowledge of which residues from RN42–56 are likely to contact the TCR. A putative binding register was assigned for the peptide based on a previous mapping study of this epitope on I-Aβ (18) and the presence of a single N that makes a suitable P1 anchor.
To test this assignment empirically, peptides with single alanine substitutions were made for each position of the proposed register. Among the assigned MHC anchors, the P9 A clearly participated fully at P6. These results were highly consistent with our assigned register.

While a P1 D is required for binding of many peptides (14), dissimilar residues can be accommodated at P1 in other ligands. Recently, the crystal structure of the CA134–146/I-A<sup>k</sup> complex has been solved and the I-A<sup>k</sup> anchor residues identified in this structure support our model of RNase/I-A<sup>k</sup> (21). In this structure, there is a non-D, H, at P1, a small hydrophobic A at P4, the same E at P6, and a small residue G at P9. At P7, there are different residues in CA/I-A<sup>k</sup> and RN<sub>42–56</sub>I-A<sup>k</sup> (W vs N). The P7 side chain fits into a shelf that is both solvent accessible and contacting the I-Ak molecule (16). Thus, this position can accommodate a wide variety of side chains, as evidenced in HEL, RN, and CA structures. Overall, our binding analysis and the HEL/I-A<sup>k</sup> and CA/I-A<sup>k</sup> crystal structures strongly support our assignment of T45 at P2, F46 at P3, H48 at P5, and L51 at P8 as the TCR contact residues of RN<sub>42–56</sub>.

FIGURE 1. Identification of the I-A<sup>g7</sup>-restricted epitope in GPI. A. The self-epitope in GPI was mapped with panel of 24 peptides spanning residues 2–558 of the 558-aa protein. The first 23 peptides are 36 aa, and each overlaps adjacent peptides by 13 residues. Splenocytes (5 × 10<sup>5</sup>/well) from P1 to P8. At P9, the naturally occurring A was replaced with a structurally dissimilar K. These substituted peptides were tested both for their ability to induce KRN T cell proliferation (Fig. 2B) and their interaction with MHC in an I-A<sup>k</sup> binding assay (Fig. 2C). In agreement with their proposed role as TCR contacts, A substitutions at the assigned P2, P3, P5, and P8 residues did not significantly reduce I-A<sup>k</sup> binding but abrogated KRN T cell recognition. Among the assigned MHC anchors, the P9 A clearly participated in binding; accordingly, the K substitution here abrogated proliferation. Substitution of the P6 and P7 anchors more modestly reduced binding and also reduced recognition, partially at P7 and fully at P6. These results were highly consistent with our assigned register.

KRN T cells recognize three of four RN<sub>42–56</sub> TCR contacts on I-A<sup>g7</sup>

Peptide library approach for defining I-A<sup>g7</sup> recognition. We sought to compare how the KRN TCR interacts with the RN<sub>42–56</sub> and GPI<sub>282–294</sub> peptides. It was impossible to align the RN<sub>42–56</sub> and GPI<sub>282–294</sub> TCR contacts with any sequence in GPI<sub>282–294</sub>; nevertheless, the shared TCR contact spacing between I-A<sup>g7</sup>, which has an α-chain in common with I-A<sup>g7</sup>, and I-A<sup>k</sup> (16, 22) suggests that I-A<sup>g7</sup> also retains the same register. Therefore, conservation of specificity was tested by determining if any of the RN<sub>42–56</sub> contacts, when transferred to a peptide with appropriate anchors, could be seen on I-A<sup>g7</sup>. The I-A<sup>g7</sup> binding groove appears to select for small nonpolar amino acids (23) but, with the exception of a preference for D or E at P9 (24), lacks a clearly defined peptide binding motif. To minimize assumptions regarding the nature of the I-A<sup>g7</sup> anchors, we initially tested a combinatorial peptide library composed of seven consecutive positions containing 20 aa, each designated by an X (Fig. 3A). To the amino terminus was added a GKKV sequence to enhance peptide solubility (25), and small nonpolar amino acids were placed at P1 and P9 flanking seven unfixed positions at P2 though P8. CHO cells transfected with I-A<sup>g7</sup> (CHO-g7) were selected as APCs because transfected CHOs had previously been used to activate mouse T cells (25) but would not present the endogenous mouse GPI Ag. When presented by CHO-g7 APCs, the X7 peptide pool failed to stimulate KRN tg T cells, implying that it did not contain activating species at a detectable concentration (Fig. 3A). For that reason, less diverse peptide pools containing TCR contacts from RN<sub>42–56</sub> were subsequently generated using the X7 sequence as a platform.

Conserved recognition of P3 and P5 on I-A<sup>g7</sup>. To narrow diversity in the X7 pool, the P3 F, P4 V, and P5 H from RN<sub>42–56</sub> in addition to an alanine at P6, were introduced into the X7 sequence. The P3 F and P5 H were chosen for transfer because KRN T cells have absolute requirement for each to recognize I-A<sup>k</sup> (later shown
in Figs. 4 and 5). The potential TCR contacts at P2 and P8 were left unfixed as was P7, which shares spacing with a presumptive TCR contact in another I-A<sup>b</sup>-binding peptide (23). When presented by CHO-g7 APCs, this pool, termed X3, strongly stimulated KRN T cells (Fig. 3A), suggesting conserved recognition at P3 and P5 between I-A<sup>k</sup> and I-A<sup>g7</sup>.

Conserved recognition of P2 on I-A<sup>g7</sup>. To determine KRN T cell specificity at P2 on I-A<sup>g7</sup>, the 20 aa at the P2 position of X3 were subdivided into five sets of 4 aa each, termed J1 through J5. Multiple amino acids are recognized at this position because all five subpools measurably stimulated KRN T cells; however, the J2 subpool induced the greatest proliferation (Fig. 3B, left). This subpool contains G, S, T, and aminobutyric acid (Abu) in place of C. To fix a single amino acid at P2, the X3 pool was individually substituted with G, S, T, or C at the P2 position. Among these subpools, only the one containing a T at P2 induced proliferation (Fig. 3B, right), demonstrating that the KRN T cell’s capacity to recognize threonine at P2 on I-A<sup>k</sup> is also conserved on I-A<sup>g7</sup>.

Identification of P7 on I-A<sup>g7</sup>. A similar substitutional analysis at P7 of the X3 pool revealed that only the subpool containing J2 at that position is stimulatory (Fig. 3C, left). Furthermore, within J2, G was the only amino acid that permitted recognition (Fig. 3C, right). This required P7 G to have the small, nonpolar features of an I-A<sup>g7</sup> anchor and could also lend necessary flexibility to the peptide backbone for positioning an adjacent TCR contact. Nevertheless, the possibility of it directly contacting the TCR cannot be excluded outright.

Altered recognition of P8 on I-A<sup>g7</sup>. The recognition of the P2, P3, and P5 contacts from RN<sub>42–56</sub>/I-A<sup>k</sup> on I-A<sup>g7</sup> strongly supports a conserved register for peptide recognition between the two MHCs. We questioned whether recognition of the fourth contact, the L at P8, might also be conserved. Therefore, a single peptide derived from X3 was generated with T at P2, G at P7, and L at P8; this ligand was termed g7RNase to reflect that it bore all four TCR contacts from RN<sub>42–56</sub>. The g7RNase peptide did not stimulate KRN T cells (Fig. 3D, right), suggesting altered recognition at P8 on I-A<sup>g7</sup>. However, multiple J subpools at P8 did stimulate (Fig. 3D, left). Most potent among these was the J3 subpool, which contains F, Y, W, and P. For that reason, four peptides were synthesized, substituting F, Y, W, or P for the P8 L in g7RNase. Among these, only peptides containing Y and F induced KRN T cell proliferation; the ligand containing Y was the more potent of the two (Fig. 3D, right) and induced proliferation at doses lower than the minimal GPI<sub>282–294</sub> concentration. To reflect its ability to mimic the recognition of RN<sub>42–56</sub>/I-A<sup>k</sup> on I-A<sup>g7</sup>, this peptide was termed g7-mimic (g7-M).
Recognition of RN42–56 and GPI282–294 is partly conserved

To understand the structural relationship among RN42–56, g7-M, and GPI282–294, we aligned them based on the information gained by generating the mimic peptide. RN42–56 and g7-M are easily aligned using their three shared TCR contacts (Fig. 4A). Remarkably, the pathogenic GPI282–294 epitope contained an H and a G at an identical spacing to the P5 H and the required P7 G in g7-M. By aligning GPI282–294 based on the identity of these two residues (Fig. 4A), the optimal P2 residue is determined by replacing the most favored subpool at P2 (J2) with each of its constituents excluding Abu, which is replaced by the naturally occurring C. C. The P3 position of the X3 pool is deconvoluted by a process identical with that for P2 in B. D. In the left panel, the P8 position of the X3 pool is analyzed by replacement with five subpools as in B. In the right panel, single synthetic peptides derived from X3 are tested for recognition by KRN T cells on I-A^e. Each peptide contains the favored residues at P2 and P7 (from B and C above) and one of the four components of the optimal subpool (J3) at P8. The most potent of these four peptides, labeled g7-M, contains three TCR contacts from RN42–56 and is compared with a peptide conserving all four contacts (g7RNase). Proliferation was measured as in A, and each assay was repeated at least three times.

GPI282–294 peptide preserved recognition, as did placement of the GPI282–294 contacts on g7-M (Fig. 4B). Furthermore, the peptide dose response was determined by the identity of the anchors and not by the TCR contacts themselves; thus, secondary effects of anchor differences on the recognition of TCR contact residues here may be excluded. These results strongly support the chosen alignment of RN42–56, g7-M, and GPI282–294.

The conservation of an H at P5 between RN42–56 and GPI282–294 suggested that this side chain may be required for recognition by the KRN TCR, irrespective of the presenting MHC molecule. To test this possibility, peptide pools containing 19 aa at P5 (X19 pools) excluding H were generated from RN42–56, g7-M, and GPI282–294. By this method, H at P5 was found to be essential for recognition of RN42–56 on I-A^k (Fig. 4C, left). Similarly, H was the only residue recognized at P5 of both g7-M and GPI282–294 on I-A^e (Fig. 4C, right). This finding clearly indicates conserved sequence recognition between a foreign peptide and an autoantigen presented by distinct MHC molecules.

Peptide recognition is partially altered between I-A^k and I-A^e

Three of four RN42–56 TCR contacts could be recognized on I-A^e, and, among these three, conservation of the P5 H was required. However, differences in which amino acids can be tolerated at the
P2, P3, and P8 TCR contacts exist between the two MHC molecules. For instance, an X19 pool based on RN 42–56 was used to show that F is uniquely recognized by KRN T cells at P3 of I-A\(^k\) (data not shown). While g7-M shares this F, the presence of an A could alter peptide recognition to approximate that on I-A\(^g\). Modified recognition of peptide seen between I-A\(^k\) and I-A\(^g\) could arise from amino acid differences in their peptide binding sites or their TCR recognition surfaces. MHC molecules vary widely in the topologies of their binding grooves but show more limited polymorphism on their surfaces (26). To reveal surface amino acid differences that may alter peptide recognition, the I-A\(^k\) and I-A\(^g\) protein sequences were compared in concert with the I-A\(^k\) crystal structure. Among the 24 aa differences between the two MHCs, five residues on the recognition surface were selected for study (Fig. 6D). This cell line thus expresses a molecule predicted to mimic the I-A\(^g\) crystal structure. Among the 24 aa differences between the two MHCs, five residues on the recognition surface were selected for study (Fig. 6D). These residues are all TCR accessible but do not contribute to the binding pockets for the peptide anchors. The 5 aa in I-A\(^k\) (a53, a65, b78, b84A, and b87) were changed to their counterparts in I-A\(^g\), and the resulting mutant I-A\(^k\) molecule (I-A\(^k\)Δ5) was expressed in M12.C3 cells for use as an APC line (C3-A\(^k\)Δ5). This cell line thus expresses a molecule predicted to mimic the I-A\(^g\) recognition surface but maintain an I-A\(^k\) binding groove.

We questioned whether the five surface changes in I-A\(^k\)Δ5 could alter peptide recognition to approximate that on I-A\(^g\). Notably, KRN T cells only weakly recognized RN 42–56 on I-A\(^k\) (Fig. 6B). Because the L at P8 of RN 42–56 is also not recognized on I-A\(^g\), this residue was excluded from a pool of peptides containing 19 other amino acids at P8 (RN 42–56 X19@P8). This peptide pool was presented by I-A\(^k\)Δ5 to produce strong KRN T cell proliferation, demonstrating a loss of leucine recognition at P8 on the mutant molecule. Furthermore, when RN 42–56 was substituted at P8 with a Y, the residue at this position in g7-M, recognition was

**Figure 4.** Partially conserved recognition of RN 42–56 and GPI 282–294. A. The RN 42–56 sequence is shown aligned with g7-M based on three shared TCR contacts. GPI 282–294 is aligned with g7-M based on shared spacing of an H and G. TCR contacts are underlined, and the conserved H and G residues are shown in bold. B. The alignment of g7-M and GPI in A is tested by transferring the putative g7-M contacts to the GPI peptide (g7-M contacts/GPI) or visa versa (GPI contacts/g7-M). T cell recognition of these peptides is assayed as in Fig. 1A. Each assay was repeated at least three times. C. The conserved amino acid differences at P2 of both RN 42–56 and GPI 282–294 (g7-M X19@P2, left) and in both g7-M and GPI 282–294 (g7-M X19@P5, GPI 282–294 X19@P5; right). The proliferative response of KRN T cells to these peptides was measured in the left panel as in Fig. 2B or the right panel as in Fig. 1A.

**Figure 5.** Modified recognition of peptide between I-A\(^k\) and I-A\(^g\). The P2, P3, and P8 TCR contacts of both RN 42–56 and GPI 282–294 were individually substituted with each of the five subpools (J1–J5) of amino acids used in Fig. 3. In this experiment, the amino acids present in the natural peptides at these positions were excluded from the substituted subpools that normally contain them, thus preventing any peptide pool from containing the wild-type ligand. Recognition of substituted RN 42–56 pools on I-A\(^k\) was measured using K/B.AKR splenocytes as in Fig. 2B. Recognition of GPI 282–294 on I-A\(^g\) was measured using K/B splenocytes and NOD splenic APCs as in Fig. 1A. The proliferative response to each peptide pool was rated relative to that of the corresponding wild-type peptide. Dose-response curves that are decreased <10-fold of wild-type are rated +++. Those diminished 10- to 100-fold are rated ++. Ones decreased 100- to 1000-fold receive a +. Curves shifted >10,000-fold are rated /+. /- and those that gave no response are indicated with a −. Each dose response was repeated at least three times.

**Discrete differences between the I-A\(^k\) and I-A\(^g\) surfaces alter peptide recognition**

The modified recognition of peptide seen between I-A\(^k\) and I-A\(^g\) could arise from amino acid differences in their peptide binding sites or their TCR recognition surfaces. MHC molecules vary widely in the topologies of their binding grooves but show more limited polymorphism on their surfaces (26). To reveal surface amino acid differences that may alter peptide recognition, the I-A\(^k\) and I-A\(^g\) protein sequences were compared in concert with the I-A\(^k\) crystal structure. Among the 24 aa differences between the two MHCs, five residues on the recognition surface were selected for study (Fig. 6D). These residues are all TCR accessible but do not contribute to the binding pockets for the peptide anchors. The 5 aa in I-A\(^k\) (a53, a65, b78, b84A, and b87) were changed to their counterparts in I-A\(^g\), and the resulting mutant I-A\(^k\) molecule (I-A\(^k\)Δ5) was expressed in M12.C3 cells for use as an APC line (C3-A\(^k\)Δ5). This cell line thus expresses a molecule predicted to mimic the I-A\(^g\) recognition surface but maintain an I-A\(^k\) binding groove.

We questioned whether the five surface changes in I-A\(^k\)Δ5 could alter peptide recognition to approximate that on I-A\(^g\). Notably, KRN T cells only weakly recognized RN 42–56 on I-A\(^k\) (Fig. 6B). Because the L at P8 of RN 42–56 is also not recognized on I-A\(^g\), this residue was excluded from a pool of peptides containing 19 other amino acids at P8 (RN 42–56 X19@P8). This peptide pool was presented by I-A\(^k\)Δ5 to produce strong KRN T cell proliferation, demonstrating a loss of leucine recognition at P8 on the mutant molecule. Furthermore, when RN 42–56 was substituted at P8 with a Y, the residue at this position in g7-M, recognition was
The failure of a similar pool excluding H at P5 to induce proliferation shows that this absolute specificity requirement is maintained on I-A<sup>k</sup><sub>D</sub><sup>5</sup>, as it is on I-A<sup>g7</sup>. Taken together, these data demonstrate that the altered recognition of peptide between I-A<sup>k</sup> and I-A<sup>g7</sup> is created by a small number of amino acid differences in their highly conserved recognition surfaces.

**Discussion**

This work explains the capacity of a single TCR to recognize a foreign peptide and an arthritic autoantigen on distinct self-restricted MHC molecules. While the KRN TCR mediates a normal foreign Ag response on I-A<sup>k</sup>, it also initiates autoimmunity on I-A<sup>g7</sup> after escaping thymic tolerance. Although these two MHCs present distinct sets of peptides, we demonstrate that a TCR contact residue directly conserved between the foreign and self peptides is requisite for the receptor’s pathogenic cross-reactivity. The spectrums of amino acids recognized at the other three positions were overlapping but partially distinct between the two MHCs. These changes in TCR recognition at other peptide contacts are explained by discrete differences in otherwise highly homologous MHC recognition surfaces and not by the disparate binding grooves. This newly defined ability to recognize multiple MHC molecules in a single animal increases the number of potentially pathogenic self-interactions available to a T cell.

The escape of KRN T cells from thymic negative selection in K/B<sub>3</sub>N mice by the ubiquitous GPI Ag remains paradoxical because NOD (H-2<sup>e7</sup>) splenic APCs readily activate T cells from K/B mice. For the I-E<sup>k</sup>-restricted 3.L2 TCR, even presentation of a low-affinity antagonist peptide still deletes efficiently in the thymus (27). Because cytoplasmic GPI would not be accessible to the MHC class II pathway, the escape of KRN T cells could be explained by insufficient levels of serum GPI to cause adequate thymic presentation. Thus, a possible fall in serum GPI level with age could explain why thymic deletion appears less efficient in K/B × N mice at 3 wk and beyond (6). Another contribution to the escape

**FIGURE 6.** Five amino acid differences between the I-A<sup>e</sup> and I-A<sup>e7</sup> surfaces alter peptide recognition. A, Shown is the solvent-accessible surface of the I-A<sup>e</sup> crystal structure (16). The RN<sub>42-56</sub> peptide, shown in gray, is modeled into the binding groove. The MHC molecule is white with the exception of five residues in black that differ in I-A<sup>e7</sup> and do not contribute to the peptide anchor binding pockets. The mutations made in I-A<sup>e</sup> to convert each residue to its I-A<sup>e7</sup> counterpart are labeled. The figure was prepared using Grasp software. B and C, The proliferative response of K/B splenocytes (5 × 10<sup>5</sup>/well) is shown to a series of substituted RN<sub>42-56</sub> peptides and peptide pools that are presented on C3-A<sup>e</sup><sub>D</sub><sup>5</sup> cells (2 × 10<sup>5</sup>/well). The single peptides include RN<sub>42-56</sub> substituted with the differing TCR contacts in GPI<sub>282-294</sub> (RN<sub>42-56/GPI contacts) or with a Y at P8 (Y@P8). Also tested was a peptide pool containing 19 aa excluding the naturally occurring L at P8 (X19@P8). Other pools contain a Y at P8 and 19 residues, excluding the natural one, at P3 or P5 (Y@P8, X19@P3; Y@P8, X19@P5). Proliferation is measured as in Fig. 1A. Each experiment was repeated at least three times.
of KRN T cells from tolerance may derive from some biochemical properties of I-A^β that distinguish it from other MHC II molecules. The I-A^β αβ heterodimer has both a shortened cell surface half life and a poor ability to retain bound peptides (28). These traits have been correlated in vivo with an elevated frequency of autoreactive T cells, which is proposed to originate from less efficient negative selection on this MHC allele (29, 30). Ineffective negative selection of autoreactive T cells is also a proposed mechanism by which RA-associated MHC alleles confer disease susceptibility (31). Thus, the failure of deletion in K/B × N mice may, at the level of a single tg TCR, model phenomena in the broader repertoires of both NOD mice and RA-susceptible humans. Moreover, certain MHC alleles that protect against collagen-induced arthritis in mice (32, 33) and RA in humans (34) could serve to delete such autoreactive components of the T cell repertoire.

The unusual biochemical properties of I-A^β have hindered determination of its peptide TCR contacts and anchors. However, multiple lines of evidence suggest that the register and spacing of peptide side chains recognized by KRN T cells on I-A^β is likely conserved on I-A^β. First, the I-A^d molecule, which shares a common α-chain with I-A^β, and I-A^β have peptide TCR contacts at identical positions (16, 22). Secondly, recent work has shown that binding of a particular peptide to I-A^β is enhanced by a D or E at P9 (24). The existence of a P9 D in our assignment of GPI further supports the validity of our binding register. With exception of this P9 position, the I-A^β binding groove readily accepts small non-polar amino acids and lacks clear anchor requirements (23), making the assigned P2 T, P3 F, P5 H, and P8 Y in the g7-M peptide unlikely MHC anchors. The P1, P4, P6, and P7 positions in g7-M and GPI282–294 have small, nonpolar side chains, which should be readily accommodated in the binding groove. Finally, the I-A^dΔ5 molecule was designed to conserve the I-A^d binding groove and yet has a TCR contact surface that converts peptide recognition to mimic that on I-A^β. Therefore, the positions of the peptide side chains relative to the adjacent features of the MHC surface are grossly conserved between I-A^d and I-A^β, placing them in the same register. As a unit, these observations lend strong support to our assignment of TCR contacts for GPI282–294 on I-A^β.

While examples of single TCRs interacting with multiple peptide:MHCs ligands existed previously (35, 36), this work provides a detailed structural comparison of an autoreactive TCR’s interactions with naturally occurring peptides on distinct MHC molecules. In contrast, the past structural studies of dual recognition have focused on alloreactivity or xenoreactivity. In addition, they have relied upon synthetic peptides derived from peptide libraries in the absence of knowledge of one of the natural ligands. Among these prior studies are those using the 2C (12, 37) and 2.102 (25) TCRs, which defined a structural basis for cross-recognition of allo- and self-restricted ligands. The biological context in which these previously studied allo-ligands are recognized by their respective TCRs is fundamentally dissimilar from KRN TCR interaction with I-A^β. 2C TCR tg T cells are negatively selected by the allo-ligand I-E^d (38); likewise, 2.102 TCR tg T cells are deleted in mice expressing I-E^β (P. Allen, unpublished results). I-A^β is not, in the same sense, an allo-ligand for KRN T cells: breeding can convert it to a self-restricted ligand for the KRN TCR because substantial numbers of KRN T cells escape thymic deletion and remain sufficiently functional to cause autoimmunity in the periphery.

The involvement of structural mimicry in dual recognition has previously been examined for a T cell clone that sees both self-restricted and xeno-MHC ligands (39). In this instance, differences in peptide recognition were observed at all TCR contacts. In concert with crystallographic data revealing limited structural similarity between the two TCR contacting surfaces (13), functional conservation was concluded to occur in the absence of structural mimicry. The TCR in fact possesses conformational flexibility that could allow it to accommodate dissimilar ligands (40). However, the absolute requirement here of a histidine at P5 implies that at least one critical TCR-peptide contact is conserved between our two complexes, revealing some direct structural conservation in our system. Also unlike the cited example of xeno-recognition, allo-recognition or dual self-restricted recognition may often occur across highly similar MHC surfaces. Peptide specificity on I-A^α is converted to resemble that on I-A^β simply by transferring five surface amino acids; thus cross-reactivity occurs here in the context of minor variations to highly similar contact surfaces. A comparable specificity shift has been documented for the 2.102 TCR by transferring 6 aa from the allo-ligand I-E^β to the highly conserved surface of I-E^β (25), and allo-recognition of Kbm3 by the 2C TCR depends on just a single amino acid difference with the self-restricted K^b (41). Thus, structurally conserved recognition of dual MHC surfaces may be a generalizable phenomenon.

It is intriguing to speculate how the ligand recognition properties of the KRN TCR might reflect general features of autoimmune recognition. The ability to interact with dual self-MHC molecules increases the number of peptides that can potentially activate a given TCR. TCRs with this property are then predicted to have an increased probability of encountering self-peptides with pathogenic consequences. For that reason, these TCRs may be proportionally overrepresented in the subset of the repertoire comprised of autoreactive T cells. TCRs that recognize a larger number of ligands are also more prone to see a pathogen-derived epitope that cross-reacts with a self-Ag and drives autoreactivity by molecular mimicry.

Just as the ability of a TCR to see multiple self-MHC molecules increases the likelihood of autoreactivity, the potential to recognize more peptides on a given MHC molecule should have the same effect. Because the 2.102 and 2C TCRs recognize their allo-ligands with decreased peptide specificity relative to self-restricted MHC (12, 25), a larger number of theoretically possible peptides should permit allo-recognition by these receptors. However, no such conclusion can readily be drawn regarding KRN T cell recognition of self-Ag on I-A^β. While amino acid requirements are less constrained at two TCR contact positions on I-A^β relative to I-A^α, peptide specificity is actually increased at P2. Thus, comparing the number of theoretical peptides that can be seen on each MHC would demand the daunting task of testing all single amino acid substitutions at three TCR contacts. Nevertheless, it remains feasible that broadened peptide specificity on I-A^β contributes to its involvement in autoreactivity.

Overall, this work has defined multiple properties of TCR interaction with a natural endogenous peptide that is a critical autoantigen in a mouse model of RA. Future work must determine which of these features are conserved for T cell autoreactivity across the broad spectrum of autoimmune disorders.

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