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Two MHC Surface Amino Acid Differences Distinguish Foreign Peptide Recognition from Autoantigen Specificity[1]

Devraj Basu, Stephen Horvath, Leigh O'Mara, David Donermeyer, and Paul M. Allen[2]

KRN T cells can recognize two self MHC alleles with differing biological consequences. They respond to the foreign peptide RN(42–56) bound to I-A^k or alternatively initiate autoimmune arthritis by interacting with a self Ag, GPI(282–294), on I-A^{g7}. Five surface amino acid differences between the two MHC molecules collectively alter which peptide side chains are recognized by the KRN TCR. In this study, it is shown that mutation of only two of these residues, α65 and β78, in I-A^k to their I-A^{g7} counterparts is sufficient to allow recognition of the TCR contacts from GPI(282–294). To provide a detailed mechanism for the specificity change, the distinct contributions of each of these two mutations to the global effect on peptide specificity were analyzed. The α65 mutation is shown to broaden the spectrum of amino acids permissible at P8 of the peptide. In contrast, the β78 mutation alone blocks KRN TCR interaction with I-A^k and requires the simultaneous presence of the α65 mutation to preserve recognition. In the presence of the α65 mutation, the β78 residue broadens peptide recognition at P3 and prevents recognition of the P8 L in RN(42–56), thus producing the observed specificity shift. These results localize the functionally relevant differences between the surfaces of two self-restricted MHC molecules to two residues that have counterbalanced positive and negative contributions to interaction with a single TCR. They highlight how subtle structural distinctions attributable to single amino acids can stand at the interface between foreign Ag responsiveness and pathogenic autoreactivity. ***The Journal of Immunology*****, 2001, 166: 4005–4011.**

The innate plasticity of the TCR-MHC interaction has been highlighted by past studies of a single TCR interacting with diverse peptides bound to a given MHC (1–4), the same peptide bound to multiple MHC alleles (5, 6), or complexes in which both peptide and MHC components differ (7–9). Such plasticity is apparent in the recognition properties of KRN T cells, which interact productively with distinct peptides on two separate MHC alleles (10). These T cells are further remarkable for their ability to initiate a cascade of events that ends in a tissue-specific autoimmune disease. The offspring of KRN TCR transgenic mice of the C56BL/6 (H-2^b) background (K/B mice) that have been crossed to nonobese diabetic (H-2^{g7}) mice all develop an inflammatory joint disease sharing multiple features with human rheumatoid arthritis (11). In these F_1 offspring (K/B×N mice), I-A^{g7} (11), B cells (12), and CD4 T cells expressing the transgenic KRN TCR (13) are all required to initiate disease. While KRN T cells from K/B mice proliferate to I-A^{g7} in the manner of an alloresponse, I-A^{g7} can be considered a self ligand in the disease setting of a K/B×N mouse. By undefined mechanisms, KRN T cells partially escape thymic and peripheral tolerance induction in K/B×N mice and react to a systemic self Ag presented by I-A^{g7}. One consequence of this I-A^{g7}-restricted interaction with B cells is the production of a serum IgG autoantibody that is sufficient to transfer the joint inflammation to normal mice (12).

The nature of the KRN TCR's dual specificity has recently been elucidated. Glucose-6-phosphate isomerase (GPI),[3] a ubiquitous cytoplasmic protein involved in glycolysis (14), has been identified as both the target of pathogenic autoantibodies in K/B×N mice and the source of a ligand recognized by KRN T cells (15). The I-A^{g7}-restricted KRN T cell epitope contained within GPI was subsequently defined as GPI(282–294) (10). However, the KRN TCR was not derived from H-2^{g7} MHC background, but rather from a B10.A(4R) mouse, which expresses I-A^k as its exclusive MHC II molecule. This TCR originated from the R28 T cell hybridoma, which is reactive to the peptide comprised of aa 42–56 of bovine pancreatic RNase (RN(42–56)) bound to I-A^k (RN(42–56)/I-A^k) (16). Thus, in the setting of an H-2$^{k/g7}$ mouse, KRN T cells have the unprecedented capacity to recognize a foreign peptide on one self-restricted MHC molecule (RN(42–56)/I-A^k) or initiate autoimmune arthritis by seeing an autoantigen on a distinct self MHC ligand (GPI(282–294)/I-A^{g7}).

Our previous work has identified the critical differences in the peptide specificity of KRN T cell recognition that distinguish RN(42–56)/I-A^k from GPI(282–294)/I-A^{g7} (10). When bound to I-A^k, the RN(42–56) peptide has four TCR-accessible side chains: T at P2, F at P3, H at P5, and L at P8. Two of these contacts, the P3 F and P5 H, cannot be substituted with any other residues without eliminating KRN T cell recognition. The ability to see additional P3 amino acids on I-A^{g7}, particularly the P3 A in GPI(282–294), is required for the recognition event that causes autoimmunity in K/B×N mice. Autoantigen and foreign Ag recognition are also distinguishable at P8, in which the L from RN(42–56) abrogates KRN T cell recognition when presented on I-A^{g7} (Table I). In contrast, altered specificity at P2 and P5 is not necessary for autoantigen recognition: the exclusive requirement of a P5 H is maintained on I-A^{g7}, while the P2 T of RN(42–56) and the P2 I of GPI(282–294) are recognized interchangeably on either MHC (see Table I). Thus, the noninterchangeable recognition of

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[3] Abbreviations used in this paper: GPI, glucose-6-phosphate isomerase; HEL, hen egg lysozyme.

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Table I. *Peptide ligands recognized by KRN T cells*

Peptide	MHC	Position/Sequence[a]																	Response
						P1	P2	P3	P4	P5	P6	P7	P8						
RN(42–56)	I-A^k			P	V	N	**T**	**F**	V	**H**	E	S	**L**	A	D	V	Q	A	Yes[b]
RN(42–56) I@P2	I-A^k			P	V	N	**I**	**F**	V	**H**	E	S	**L**	A	D	V	Q	A	Yes[c]
g7RNAse	I-A^{g7}	G	K	K	V	A	**T**	**F**	V	**H**	A	G	**L**	G					No[b]
g7-M	I-A^{g7}	G	K	K	V	A	**T**	**F**	V	**H**	A	G	**Y**	G					Yes[b]
GPI (282–294)	I-A^{g7}				L	S	**I**	**A**	L	**H**	V	G	**F**	D	H	F	E		Yes[b]

[a] TCR contact positions are underlined. TCR contact residues are shown in bold.
[b] Ref. 10.
[c] Unpublished data.

peptide TCR contacts between RN(42–56)/I-A^k and GPI(282–294)/I-A^{g7} derives from altered interaction of KRN T cells with the P3 and P8 positions.

We sought to define the precise contributions of individual amino acid differences between I-A^k and I-A^{g7} to the shift in peptide recognition at P3 and P8 that discriminates foreign from self. Prior work had narrowed which structural differences between I-A^k and I-A^{g7} may modify peptide recognition at P3 and P8 (10). The recent solving of the I-A^{g7} crystal structure has now greatly facilitated our ability to compare the I-A^k and I-A^{g7} molecules at a molecular level (17, 18).

Previously, five nonconserved amino acids on the I-A^k recognition surface had been mutated to their counterparts in I-A^{g7} to generate a hybrid MHC, termed I-A$^k\Delta5$, that was designed to approximate the I-A^{g7} surface, but maintain an I-A^k-binding groove. The five amino acid changes allowed efficient recognition of the TCR contacts from GPI(282–294) on the mutant I-A$^k\Delta5$ molecule in the presence of appropriate MHC anchors for I-A^k (10). Thus, the altered recognition of peptide derives from discrete differences on the MHC recognition surfaces and not the disparate binding grooves. In this study, we define a subset of the five residues that is sufficient to account for the observed shift in peptide recognition. Additional experiments dissect the distinct contributions of each residue to the global effect on peptide specificity. These results precisely localize functionally relevant differences between the surfaces of two self-restricted MHC molecules recognized by a single TCR.

Materials and Methods

Mice

K/B mice (gift, D. Mathis/C. Benoist, Joslin Diabetes Center, Boston, MA) were rederived and maintained in our colony by breeding to C57BL/6. The KRN TCR transgene was screened using PE anti-mouse CD4 and FITC anti-mouse Vβ6 (PharMingen, San Diego, CA) dual FACS staining of peripheral blood. K/B.AKR mice were derived by breeding to the congenic B6.AKR (H-2^k) background for two generations and screening blood by FACS for absence of K^b expression using a biotinylated anti-mouse H-2 K^b 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. The peptide pools contained equimolar ratios of substituent amino acids at unfixed positions, and these positions were double coupled during synthesis. All single sequence peptides were purified by HPLC, and their composition was confirmed by mass spectrometry and amino acid analysis. Peptide sequences are represented with standard single letter code. The letter X represents a mixture of 19 natural amino acids plus α-aminobutyric acid in place of C.

Generation of I-A^k and I-A^{g7} expression constructs

The I-A$^k\alpha$- and I-A$^k\beta$-chains were changed in their expression vectors, pcDNA3.1neo-A$^k\alpha$ and pcDNA3.1neo-A$^k\beta$ (gift, E. R. Unanue, Washington University, St. Louis, MO), using PCR mutagenesis, as previously described (10). The resulting vectors were named pcDNA3.1neo-A$^k\alpha$A65 and pcDNA3.1neo-A$^k\beta$A78. Both mutant chains were fully sequenced.

The I-A$^d\alpha$- and I-A$^{g7}\beta$-chain coding sequences were cloned into pcDNA3.1neo$^+$ and pcDNA3.1zeo$^+$ expression vectors (Invitrogen, Carlsbad, CA) to generate the plasmids pcDNA3.1neo-A$^d\alpha$ and pcDNA3.1zeo-A$^{g7}\beta$, as previously described (10). The position 65 A in the α-chain was mutated to a T using PCR mutagenesis, as described (10), with the following primers: 5′-ACAGAAAAACACAACTTGGGAATCTTGAC-3′ (coding); 5′-TGCTATGTTCTGCAGTCCACCTTGG-′3′ (noncoding). The resulting vector was named pcDNA3.1neo-A$^d\alpha$T65, and its insert was fully sequenced.

Generation of transfected cell lines

DAP.3 cells were cotransfected with the pcDNA3.1neo-A$^d\alpha$ and pcDNA3.1zeo-A$^{g7}\beta$ vectors using the CellFECTIN liposomal reagent (Life Technologies, Gaithersburg, MD) as per manufacturer 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 transfected with pcDNA3.1neo-A$^k\alpha$, pcDNA3.1neo-A$^k\beta$, pcDNA3.1neo-A$^k\alpha$A65, and/or pcDNA3.1neo-A$^k\beta$A78 plasmids, as appropriate to the cell line desired. Electroporation, growth, and selection were performed as previously described (10).

Drug-resistant transfected DAP.3 or M12.C3 cells were stained for surface MHC expression using biotin-10.3.6 (anti-mouse I-A$^{k/g7}\beta$; PharMingen) or biotin-11.5.2 (anti-mouse I-A$^k\alpha$; PharMingen) plus PE-streptavidin; they then were sorted on a FACSVantage instrument (Becton Dickinson, Mountain View, CA).

Proliferation assays

Assays using transfected DAP.3 cells as APCs were performed in DMEM medium supplemented with 10% FCS, 1 mM Glutamax (Life Technologies), and 50 μg/ml gentamicin. Assays using transfected M12.C3 cells or mouse splenic APCs were performed in RPMI 1640 medium containing 10% FCS, 5×10^{-5} M 2-ME, 1 mM Glutamax, and 50 μg/ml gentamicin.

Before use, APC cell lines 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 flat-bottom tissue culture plates (Costar, Cambridge, MA), which were pulsed at 48 h with 0.4 μCi [^{3}H]thymidine and harvested 18–24 h later, as described (19). Proliferation is expressed as counts incorporated (average of duplicate wells).

Modeling of the GPI(282–293)/I-A^{g7} structure

A model of the GPI(282–293)/I-A^{g7} was generated using the InsightII suite of programs (Molecular Simulations, San Diego, CA) based on the hen egg lysozyme (HEL)(11–25)/I-A^{g7} crystal structure (17) and involved replacement of 12 side chains. Each of the side chains was positioned individually into the lowest energy rotamer. Energy minimization studies, performed using the Discover module software (Molecular Simulations), indicated that the model was energetically favorable. The model of RNase(42–56)/I-A^k, as previously published, was used for Fig. 1*A* (10).

Results

Two surface differences between I-A^k and I-A^{g7} are next to the P3 and P8 peptide positions

Of the 24 amino acid differences between I-A^k and I-A^{g7}, five positions (α53, α65, β78, β84A, and β87) had previously been

selected for study based on their accessibility to TCR recognition and lack of contribution to the I-A^k peptide-binding pockets (20). Because changing these five residues in I-A^k to their I-A^{g7} counterparts (to create I-A$^k\Delta5$) allows recognition of the TCR contacts from GPI(282–294) (10), we sought a more refined understanding of how the residues alter specificity. Of the five mutations, two are located adjacently to the P3 and P8 peptide positions (Fig. 1*A*), where the context of the presenting MHC molecule differentiates KRN T cell specificity for RN(42–56) and GPI(282–294). Notably, both of these residues are clustered near the center of the recognition surface, where the peptide's TCR contacts reside, while the other three mutations lie more peripherally. In I-A^k, one of these two, αT65, is next to the P8 residue of the peptide and makes van der Waals contacts with P6, P7, and P8. The other, βV78, lies adjacent to the P3 side chain and has van der Waals interactions with P2, P3, and P4 (20). Comparison of I-A^k with the recent I-A^{g7} crystal structures (17) reveals minimal differences in the TCR recognition surface, with α65 and β78 having a very similar spatial relationship to the P3 and P8 peptide positions in both MHCs (Fig. 1, *A* and *B*). Since alterations in peptide specificity at P3 and P8 differentiate GPI(282–294)/I-A^{g7} recognition, αA65 and βA78 were considered likely candidates to produce those changes.

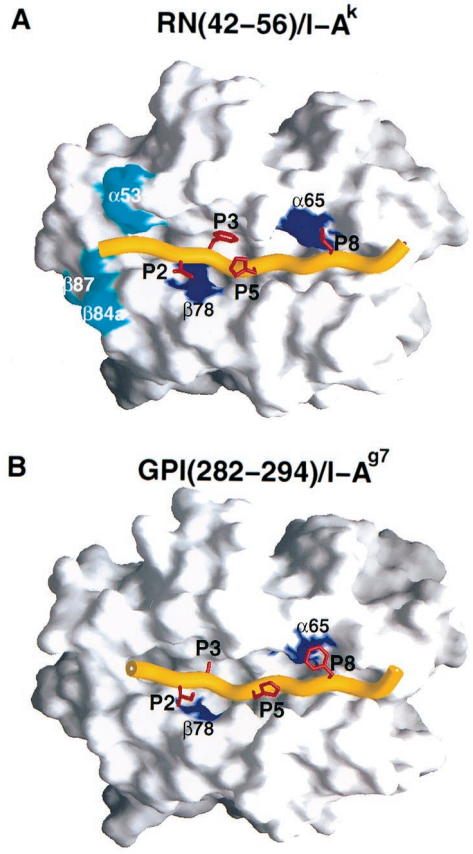

FIGURE 1. Comparison of the I-A^k and I-A^{g7} surfaces. The solvent-accessible surfaces of the I-A^k crystal structure (*A*) (20) and I-A^{g7} (17) (*B*) are shown. The RN(42–56) peptide (*A*) (amino acid sequence = PVNTFVHESLADVQA) has been modeled into the binding groove to replace the solved HEL(50–62) peptide structure, as previously described (10). The peptide backbone is shown as a yellow worm. The side chains of the four TCR contact residues, P2 = T, P3 = F, P5 = H, and P8 = L, are shown in red. The I-A^k molecule is white, with the exception of five colored surface residues that have been changed to their I-A^{g7} counterparts in I-A$^k\Delta5$. Two of these mutated positions, α65 and β78, are distinguished in purple as the sites of changes present in I-A$^k\Delta2$. The α65 and β78 amino acids reside in the central region of the MHC molecule, adjacent to the respective P8 and P3 peptide positions. The GPI(282–294) peptide (*B*) (amino acid sequence = LSIALHVGFDHFE) has been modeled into the binding groove to replace the solved HEL(11–25) structure (17). The same coloring scheme as described in *A* was used. The four TCR contact residues are P2 = I, P3 = A, P5 = H, and P8 = F. The figure was prepared using Grasp software (http://honiglab.cpmc.columbia.edu/grasp/).

Mutating α65 and β78 in I-A^k allows recognition of the GPI(282–294) TCR contacts

Based on their location on the I-A^k recognition surface, it was hypothesized that the αA65 and βA78 mutations account for the KRN TCR's shift toward I-A^{g7}-like recognition specificity on I-A$^k\Delta5$. To test this possibility, a cell line (C3-A$^k\Delta2$) was generated to express an I-A^k molecule containing only these two mutations (I-A$^k\Delta2$).

The two mutations in I-A$^k\Delta2$ were tested for their ability to confer the shift in P8 specificity between I-A^k and I-A^{g7}. KRN T cells are able to see identical P2, P3, and P5 contacts on both MHCs. However, they fail to recognize the P8 L from RN(42–56)/I-A^k when transferred to a peptide on I-A^{g7}, instead responding to a Y in its place (Table I). This change in specificity on I-A^{g7} is mimicked equally well by I-A$^k\Delta5$ and I-A$^k\Delta2$: KRN T cells saw the P8 L of RN(42–56) on neither MHC, while a peptide pool containing 19 other amino acids at P8 (RN(42–56) X19@P8) restored recognition of both, as did a Y at P8 (RN(42–56) Y@P8) (Fig. 2).

Another feature that distinguishes KRN T cell recognition of the two MHCs is peptide specificity at P3. The KRN TCR exclusively requires an F at P3 and an H at P5 to recognize RN(42–56)/I-A^k. While I-A^{g7} maintains the P5 requirement, several other amino acids are permissible at P3 (10). Likewise, a pool containing 19 aa at P3, excluding F, was comparably recognized on I-A$^k\Delta5$ and I-A$^k\Delta2$ (Fig. 2), revealing degenerate recognition at P3 on both mutant ligands. The failure of a similar pool excluding H at P5 to induce proliferation shows that this absolute specificity requirement is equally maintained on I-A$^k\Delta5$ and I-A$^k\Delta2$. When taken together, these results demonstrate that the αA65 and βA78 mutations in I-A$^k\Delta2$ are alone sufficient to account for the capacity of I-A$^k\Delta5$ to mimic peptide recognition on I-A^{g7}.

Most importantly, KRN T cells recognize GPI(282–294) as an autoantigen on I-A^{g7}, but fail to respond to the GPI peptide's TCR contacts substituted into the RN(42–56) sequence (RN(42–56)/GPI contacts) when presented by I-A^k (Fig. 2). Recognition of these GPI TCR contacts is reconstituted by both the five mutations in I-A$^k\Delta5$ and the subset of two changes in I-A$^k\Delta2$. Thus, the mutually exclusive recognition of the peptide TCR contacts in RN(42–56)/I-A^k and GPI(282–293)/I-A^{g7} is sufficiently explained by two surface amino acid differences between the MHC molecules.

A single αA65 mutation allows recognition of more residues at P8 of the peptide

The αA65 mutation had previously been reported to convert I-A^k to an allo-ligand for the KRN TCR (16); however, this apparent alloreactivity was later seen to be dependent upon an FCS-derived factor, since recognition was eliminated by growing cells expressing I-A$^k\alpha$A65 in serum-free medium (21). To study the isolated impact of αA65 on I-A^k recognition, we expressed I-A$^k\alpha$A65 in M12.C3 cells to derive the cell line C3-A$^k\alpha$A65. Growing C3-A$^k\alpha$A65 in mouse serum in place of FCS prevented direct recognition by KRN T cells (Fig. 3*A*) and provided a low background APC line upon which the recognition of exogenous peptides could be tested.

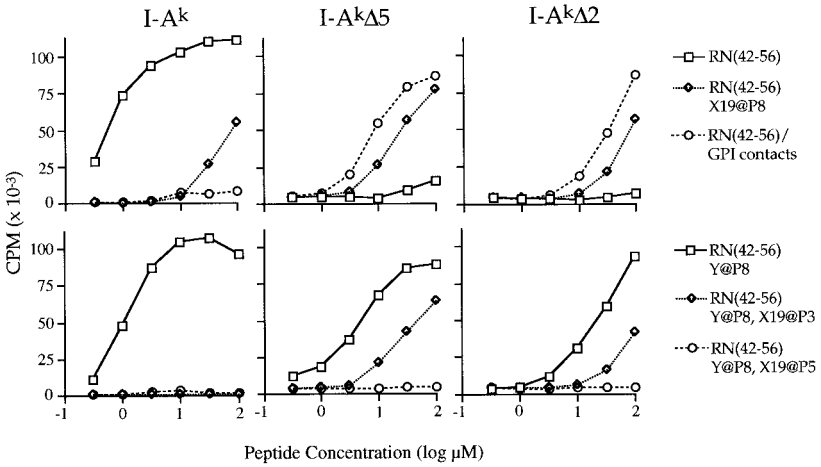

FIGURE 2. Differences at α65 and β78 alter peptide recognition between I-A^k and I-A^{g7}. To assay recognition of I-A$^k\Delta$5 or I-A$^k\Delta$2, splenocytes (5×10^5/well) from KRN TCR transgenic mice on a C57BL/6 background (K/B mice) were added to C3-A$^k\Delta$5 cells or C3-A$^k\Delta$2 cells (2×10^5/well) in the presence of peptide. To assay recognition of I-A^k, splenocytes (5×10^5/well) from K/B mice bred on to the congenic B6.AKR (H-2^k) background (K/B.AKR mice) were incubated with each peptide. The proliferative response is shown to a series of substituted RN(42–56) peptides and peptide pools. These include RN(42–56) substituted with the differing TCR contacts in GPI(282–294) (RN(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 naturally occurring one, at P3 or P5 (Y@P8, X19@P3; Y@P8, X19@P5). Proliferation was measured as [^{3}H]thymidine incorporation after pulsing at 48 h and harvesting 16–24 h later. The data are representative of at least three independent experiments, and values represent the average of duplicate cultures. Range of the duplicate values was <20% of the average.

The location of αT65 adjacent to P8 in the I-A^k crystal structure (20) suggested that the αA65 mutation could be responsible for altering peptide recognition at that position. Therefore, the influence of the αA65 mutation on P8 recognition was tested comprehensively using 20 peptides, each with a different amino acid substitution at P8 (Fig. 3*B*). All seven of the residues seen by KRN T cells at P8 on I-A^k (F, Y, L, D, E, N, Q) were also recognized on I-A$^k\alpha$A65. The preservation of L recognition at P8 on I-A$^k\alpha$A65 establishes that βA78 must contribute to its loss of recognition on I-A$^k\Delta$2. Importantly, 11 other P8 residues (I, V, A, M, G, S, T, C, P, R, H) that gave no response on I-A^k were recognized on I-A$^k\alpha$A65. The αA65 mutation thus markedly broadens specificity at P8, making peptide recognition by the KRN TCR more degenerate. Because there was not a loss of L recognition at P8 on I-A$^k\alpha$A65, as is demonstrable on I-A$^k\Delta$2 and I-A^{g7}, the αA65 single substitution cannot alone explain the total effect on peptide recognition.

To test whether αA65 altered peptide recognition at the three other TCR contacts of RN(42–56), a series of peptide pools were generated by making multiple substitutions at P2, P3, and P5. Five peptide pools (J1-J5) were synthesized for each of these positions, with each pool containing four different amino acids at the appropriate position. Notably, the wild-type residue at the substituted position was excluded so that no pools contained unsubstituted RN(42–56). These pools were presented by I-A^k or I-A$^k\alpha$A65 and compared with RN(42–56) for their ability to stimulate KRN T cells (Fig. 4). At P2 of both I-A$^k\alpha$A65 and I-A^k, all five peptide pools gave dose responses within 10-fold of RN(42–56); thus, no effect of αA65 on P2 recognition was readily apparent. At P5, none of the five peptide pools was recognized on either MHC, demonstrating that the absolute requirement for a P5 H on I-A^k is maintained in the presence of the αA65 mutation. Although marginally detectable responses to two P3 pools on I-A$^k\alpha$A65 were documented at high concentrations, the exclusive requirement of a P3 F for recognition of I-A^k was also largely retained in the presence of αA65. Thus, the αA65 mutation does not prominently alter recognition at P2, P3, or P5. The absence of significant degeneracy at P3 on I-A$^k\alpha$A65 indicates that βA78 must participate in broadening P3 specificity on I-A$^k\Delta$2.

βA78 alone blocks I-A^k recognition by KRN T cells, but in the presence of αA65 alters peptide specificity

To determine the isolated effect of the βA78 mutation, an I-A^k molecule containing this single amino acid change (I-A$^k\beta$A78) was expressed in M12.C3 cells to generate an APC line (C3-A$^k\beta$A78). Although C3-A$^k\beta$A78 and C3-A$^k\Delta$5 had comparable surface levels of their MHC α- and β-chains (Fig. 5*A*), none of the peptides or peptide pools that KRN T cells recognize on I-A^k or I-A$^k\Delta$2 (Fig. 2) were seen on I-A$^k\beta$A78. The abrogated responses to RN(42–56), RN(42–56)/GPI contacts, and RN(42–56) X19@P8 are displayed as examples (Fig. 5*B*). Thus, introduction of the solitary βA78 mutation into I-A^k hinders productive interaction by the KRN TCR with the MHC surface. The concomitant presence of αA65 in I-A$^k\Delta$2 must therefore compensate for this negative effect of βA78 and restore recognition. Thus, while βA78 blocks I-A^k recognition as an isolated mutation, it is essential to the shift in peptide specificity in the presence of αA65: amino acids other than just F are accommodated at P3, and recognition of L is prevented at P8.

αA65 participates in autoimmune recognition of GPI(282–294)/I-A^{g7}

In the presence of βA78, αA65 becomes a crucial second mutation for preserving I-A^k recognition; thus, the αA65 residue may also play a critical role in autoimmune recognition of I-A^{g7} in K/B$\times$N mice. To test this possibility, the mouse fibroblast line DAP.3 was transfected with wild-type I-A^{g7} or a mutant I-A^{g7} in which αA65 had been replaced with the αT65 derived from I-A^k (I-A$^{g7}\alpha$T65). The two cell lines, DAP.3-A^{g7} and DAP.3-A$^{g7}\alpha$T65, expressed comparable levels of surface I-A^{g7} by FACS (Fig. 6*A*), but KRN T cells did not recognize the g7-M peptide (see Table I) nor the GPI(282–294) autoantigen in the presence of the αT65 mutation (Fig. 6*B*). This effect is not likely attributable to a loss of peptide binding by I-A$^{g7}\alpha$T65 since the mutation did not abrogate the

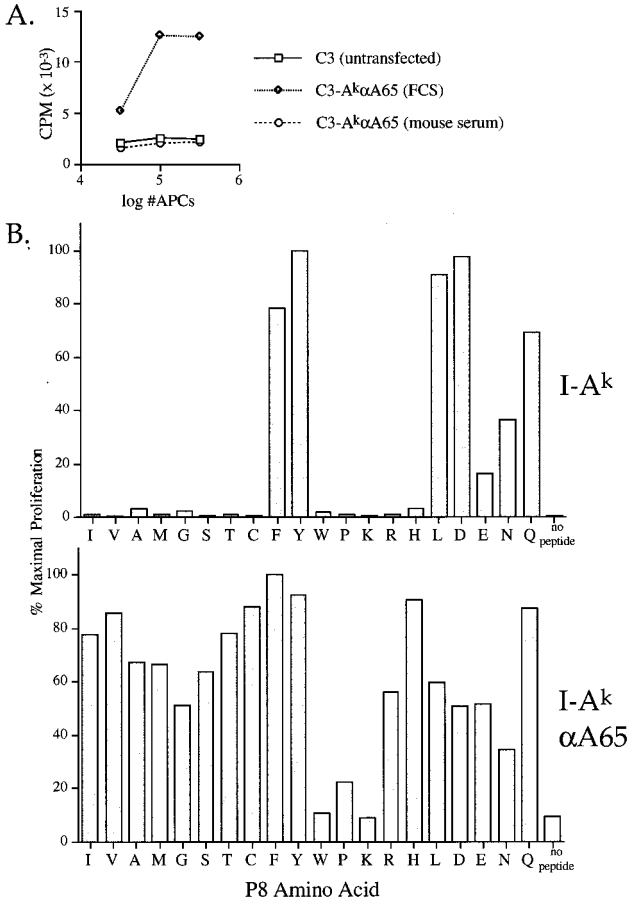

FIGURE 3. The αA65 mutation decreases peptide recognition specificity at P8. *A*, K/B splenocytes were incubated in the presence of the indicated number of M12.C3 (C3) cells or C3-A^{k}αA65 APCs grown in medium containing either 10% FCS or 0.8% mouse serum. The function of each APC was assayed in the same medium in which it was grown. The proliferative response of K/B splenocytes was measured as in Fig. 2, and data are representative of two identical experiments. *B*, RN(42–56) was substituted at P8 with each of the 20 indicated natural amino acids. These peptides were tested for recognition on I-A^k using K/B.AKR splenocytes (5×10^5/well) or on I-A^{k}αA65 using C3-A^{k}αA65 APCs (2×10^5/well) as in Fig. 2. The proliferative response is shown for a high dose of the peptide (31.6 μM), and is depicted as a percentage of the maximal response for any peptide at that dose. Proliferation was measured as in Fig. 2, and data are representative of three independent experiments. Values represent the average of duplicate cultures, and the range of duplicate values was <20% of the average.

response of a second I-A^{g7}-restricted T cell clone (22) that is specific for HEL (11–25) (data not shown). Thus, α65 is the site of a functionally critical difference between the surface topologies of I-A^k and I-A^{g7}: it not only diversifies the peptide specificity of KRN T cells across the two MHCs, but also is crucial for the pathogenic KRN T cell response to GPI autoantigen on I-A^{g7}.

Discussion

This study describes how amino acid differences between two MHC surfaces create a TCR's dual specificity for foreign Ag on I-A^k and arthritic autoantigen on I-A^{g7}. We propose that the α65 A in I-A^{g7} enhances the TCR-MHC interaction relative to the corresponding T in I-A^k and, in the process, creates more degenerate peptide recognition at P8. In contrast, the β78 A in place of a V diminishes the interaction such that recognition can be preserved only when the αA65 residue is simultaneously present. The hampering of recognition by βA78 may secondarily reduce the degeneracy introduced by αA65, thus blocking productive interaction with L at P8. In the presence of αA65, βA78 also introduces degenerate peptide recognition at the adjacent P3 position. Thus, our results furnish a detailed mechanism of two counterbalancing MHC surface differences that underlies a single TCR's pathogenic dual specificity for distinct self MHC molecules. They further illustrate how minimal structural variations can create diversity in peptide TCR contact recognition across highly related MHC surfaces.

The locations of the α65 and β78 residues on the MHC recognition surface suggest the manner in which they determine peptide recognition specificity. The interaction of TCRs with MHC I takes place in a consistent orientation in the existing co-crystal structures, with the TCR Vα region contacting the MHC α2 domain and the TCR Vβ recognizing the MHC α1 helix in all cases (23–26). In these instances, the TCR docks in a diagonal binding mode traversing the center of the MHC I recognition surface, with the complementarity-determining 3 regions contacting peptide side chains protruding from the MHC's binding cleft (25, 26). While multiple mapping studies suggest a similar orientation to TCR interaction with MHC II (27–29), the single existing TCR-MHC II co-crystal structure shows that long axes of the TCR and MHC recognition surfaces can also contact in an orthagonal orientation (30). Importantly, the α65 and β78 residues fall centrally in this predicted TCR footprint on I-A^k in either binding mode, while the other three previously mutated residues (α53, β84A, β87) most likely reside at the periphery or outside the footprint entirely. Thus, among the five previously mutated amino acid differences between I-A^{g7} and I-A^k (10), α65 and β78 are uniquely poised to influence peptide recognition, either by directly contacting the TCR or by influencing the conformation of adjacent peptide contacts. Accordingly, the β78 and α65 residues reside adjacent to the respective P3 and P8 peptide positions, where recognition is critically altered between I-A^k and I-A^{g7}. However, any mechanism to account for the roles of α65 and β78 must also explain their effects on peptide recognition at positions beyond their immediate vicinity. In particular, mutating β78 in I-A^k not only broadens side chain specificity at the adjacent P3, but also blocks recognition of L at the more distant P8 position. Such long-range effects are most simply explained by potential effects of the mutations on the overall efficacy of the TCR-MHC interaction. A single αA65 mutation in I-A^k may enhance binding of this molecule to the KRN TCR, thus allowing less stearically favored amino acid side chains to be accommodated at P8 and making peptide recognition more degenerate. The alanine substitution at α65 may actually allow many more side chains at P8 to exist in a conformation that contributes binding energy to the TCR-MHC interaction. While I-A^{k}αA65 still relies upon exogenous peptide to activate KRN T cells, the acquired ability of a transgene expressing this mutant molecule to delete KRN T cells in the thymus (13) is further consistent with the proposed enhancement of the interaction. In contrast, a single βA78 mutation may diminish the strength of the interaction below an activation threshold, and this decrease could be compensated by the simultaneous presence of αA65. The βA78 residue could then reverse any positive effect of αA65 upon recognition and, in doing so, further prevent the recognition of L at the distant P8 position. Such compensatory effects between dual MHC mutations are reminiscent of a prior study, in which mutating a second position of the peptide itself could offset the negative effect of a separate peptide substitution upon recognition (31).

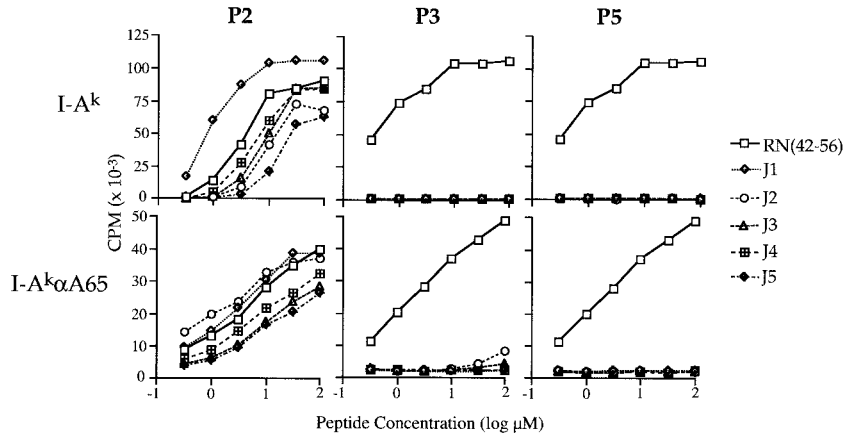

FIGURE 4. The αA65 mutation does not affect peptide recognition at P2, P3, or P5. I-A^k and I-A^kαA65 recognition by KRN T cells were determined as in Fig. 3 over a concentration range of the indicated peptide pools. The P2, P3, and P5 positions of RN(42–56) were each replaced by five pools containing four amino acids each (J1-J5). The pools used are as follows: J1 = [I, V, A, M]; J2 = [G, S, T, Abu]; J3 = [F, Y, W, P]; J4 = [K, R, H, L]; J5 = [D, E, N, Q]. Notably, the naturally occurring amino acid at a given position in RN(42–56) was excluded such that one of the five pools for each position contained only three amino acids. Data are representative of four independent experiments. Values represent the average of duplicate cultures, and the range of duplicate values was <20% of the average.

On another level, the individual and joint effects of the αA65 and βA78 mutations may be explained by their influence on conformational changes in the TCR that are required for ligand recognition. Crystallographic studies have revealed large conformational adjustments can occur in the TCR's Ag binding site upon docking with a peptide-MHC complex (23). The KRN TCR's recognition of RN(42–56)/I-A^k could then be accompanied by a conformational change that facilitates its interactions with the TCR contacts of RN(42–56), but not those of GPI(282–294). In this context, the βA78 mutation may create an MHC surface incapable of inducing a conformational state in the KRN TCR that can accommodate the TCR contacts of either the foreign or the self peptide. The joint addition of αA65 to I-A^k would then induce a TCR conformation that is selective for the GPI(282–294) contacts and approximates the structural state of the KRN TCR when bound to GPI(282–294)/I-A^{g7}.

Peptide sequence recognition by the KRN TCR is at least partially independent of the MHC anchors involved in peptide presentation. The αA65 and βA78 mutations allow KRN T cells to recognize the TCR contacts in GPI(282–294) whether they are presented on I-A^k or I-A^{g7}, despite the disparate peptide-binding requirements of these two MHCs. Past studies have documented negative effects of anchor residue substitutions (32) and changes in the chemical nature of the backbone itself (33) on TCR recognition

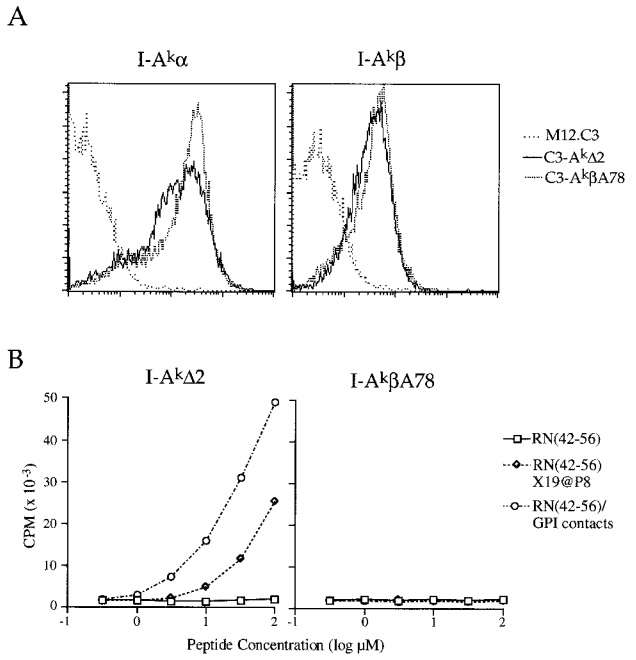

FIGURE 5. The βA78 mutation blocks recognition of I-A^k by KRN T cells. *A*, The level of I-A^k surface expression by the indicated cell lines was quantitated by FACS relative to an untransfected (M12.C3) control. *B*, The proliferative response of KRN T cells to the indicated peptides on I-A^kβA78 and I-A^kΔ2 was determined. K/B splenocytes (5 × 10^5/well) were incubated in the presence of C3-A^kβA78 or C3- I-A^kΔ2 APCs (2 × 10^5/well). Proliferation was measured as in Fig. 2. Values represent the average of duplicate cultures, and the range of duplicate values was <20% of the average.

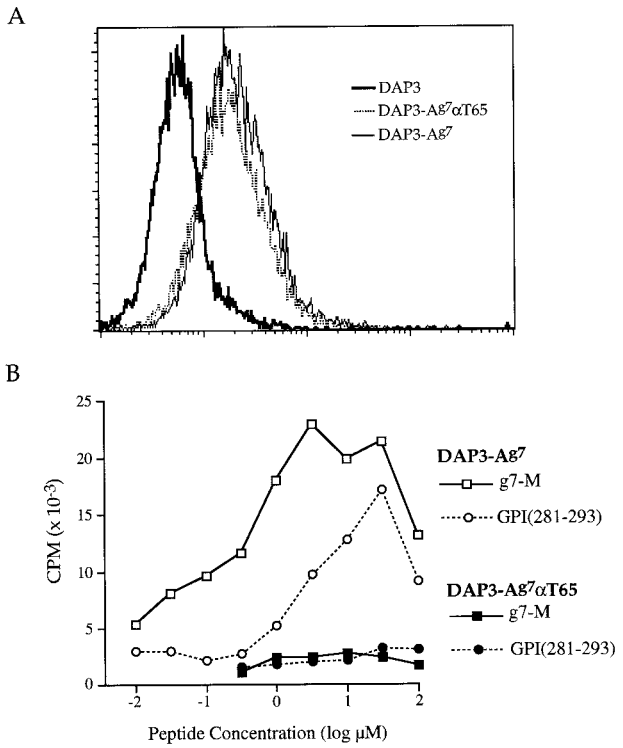

FIGURE 6. αA65 is critical for I-A^{g7} recognition by KRN T cells. *A*, The level of I-A^{g7} surface expression by the indicated cell lines was quantitated by FACS relative to an untransfected (DAP.3) control. *B*, The proliferative responses of KRN T cells to GPI(282–293) or g7-M (a synthetic I-A^{g7} bound peptide that stimulates KRN T cells; Table I) were determined on I-A^{g7} and I-A^{g7}αT65. K/B splenocytes (5 × 10^5/well) were incubated in the presence of DAP.3-A^{g7} (A^{g7}) or DAP.3-A^{g7}αT65 (A^{g7}αT65) APCs (1.5 × 10^5/well). Proliferation was measured as in Fig. 2.

in the absence of any reduction in peptide-MHC interaction. However, sequence differences at non-TCR contacts in RN(42–56) and GPI(282–294) do not appear to have a critical role in differentiating recognition of these two peptides. TCR recognition and MHC binding thus appear to be experimentally separable entities in at least some instances for certain peptides.

The degree of structural conservation suggested to be involved in dual MHC recognition has varied widely among previous studies. At one extreme, very little similarity in surface topology was reported between the crystal structures of a self-restricted MHC and a xeno-ligand recognized by a murine CTL clone (34). In contrast, the differences in the peptide specificity of a T cell clone for self I-E^k vs allo-I-E^p have been narrowed to the six amino acid differences between their α helices (9). At an opposite extreme, a single amino acid change appears sufficient to convert the self-restricted K^b to an allo-ligand for the 2C TCR (8). It merits emphasis that α65 and β78 are not the only locations on the solvent-accessible surface, where differences exist between I-A^k and I-A^{g7}; yet these two amino acids are sufficient to engender the shift in peptide recognition seen between I-A^k and I-A^{g7}. Their uniqueness is underscored by a series of other alanine substitutions that had previously been made across the length of the I-$A^k\alpha$-chain's α helix without impacting recognition by the KRN TCR (16). Thus, only a small number of polymorphic surface positions across the diverse spectrum of MHC alleles may participate in diversifying peptide recognition by TCRs that see multiple MHCs.

In summary, this work offers insight into the detailed structural mechanisms by which minor differences across highly conserved MHC surfaces can diversify peptide recognition specificity. In the case of the dual specific KRN TCR, two such amino acid differences stand between its recognition of the TCR contacts of a foreign Ag and those of a self peptide that participates in the pathogenesis of autoimmune arthritis. The capacity to interact with multiple self MHCs in this manner increases the number of potentially pathogenic self interactions available to a T cell. One can envision that a T cell with such properties might be more likely to become an instigator of an autoimmune process.

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