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Local structure of a peptide contact site on A_{α}^k

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Abstract

We have sought to determine how much amino acid diversity is tolerable at position 69 of the A_{α}^k chain, a position previously implicated as a peptide contact site. Slot-machine mutagenesis was used to create a set of 11 mutant A_{α}^k cDNAs, each specifying a different amino acid at position 69. These cDNAs were individually expressed in L cells together with a wild-type A_{β}^k cDNA to produce a panel of mutant antigen-presenting cell lines. The ability of each member of this panel to present a hen egg lysozyme and a bovine ribonuclease peptide to various T hybridomas was assessed. We found that a surprising degree of amino acid diversity is tolerable at A_{α}^k position 69: even charged (Glu, Arg) or bulky (Trp, Tyr) residues can be accommodated without abrogating cell-surface expression of A_{α}^k , peptide binding to it, or T cell recognition of it. We discuss the implications of these findings for models of T cell recognition of the class II molecule/antigen duplex.

T cell receptors only recognize Ag in the context of an MHC class I or class II molecule. The exact nature of the TCR–MHC molecule–Ag ternary complex remains somewhat enigmatic, although some major advances in our understanding have taken place over the past several years. The image of a class I molecule derived from the crystal structures of HLA-A2 (1,2) and HLA-Aw68 (3) has provided a framework for interpreting functional data (4). It has also prompted Brown *et al.* to model the antigen binding site on a class II molecule (5). Their model predicts that the class II α_1 and β_1 domains associate in a manner very similar to the class I α_1 and α_2 domains: an antigen-binding groove is formed by an alpha-helix from the α chain, an alpha-helix from the β chain and underlying beta-sheets from both. Most polymorphic residues are located within this Ag-binding groove, and their side chains generally point either into or up from the groove, as might be expected for Ag and TCR contacts, respectively.

Alanine-scan mutagenesis of the alpha-helix on the A_{α}^k chain has recently provided substantial support for the model of Brown *et al.* (6). For example, the interpretation of data from diverse functional assays converged to implicate several 'in-pointing' residues as direct peptide contact sites. Let us consider the case of residue 69. First of all, an Ala substitution at this position had a drastic effect on the presentation of hen egg lysozyme (HEL) and bovine pancreatic ribonuclease (RNase) peptides to all of

the T hybridomas analyzed (at that time), save one that appeared to recognize its cognate peptide with much lower affinity than the others. Second, Ala replacement of the usual Thr at position 69 provoked one of the hybridomas, normally specific for an RNase peptide, to recognize the A_{α}^k complex alloreactively. This observation was considered in the context of the recent evidence that many alloreactive T cells actually respond to a peptide in the groove of the MHC molecule (for discussion and references, see 7). And third, the peptide RNase 43–56 was not recognized in the context of an A_{α}^k complex carrying a mutation at α chain position 69, but an analogue of this peptide bearing a single amino acid replacement was seen quite effectively. Together, these three observations argued forcefully that the residue at this position can directly interact with peptide antigens (but see more extensive discussion in 6).

Having identified in this manner several residues likely to be peptide contact sites (6), we wondered about the structure of such critical locales and about the chemical interactions that might be in play. Would Ag binding to the MHC molecule and TCR recognition of the resulting MHC molecule/Ag duplex be tolerant of local structural perturbations? Or would either show the exquisite specificity we previously demonstrated for antibody recognition of the A_{α} chain (8)? To address this issue, we have created a panel of mutant antigen-presenting cell (APC) lines, each expressing an A_{α}^k complex with a different amino acid at

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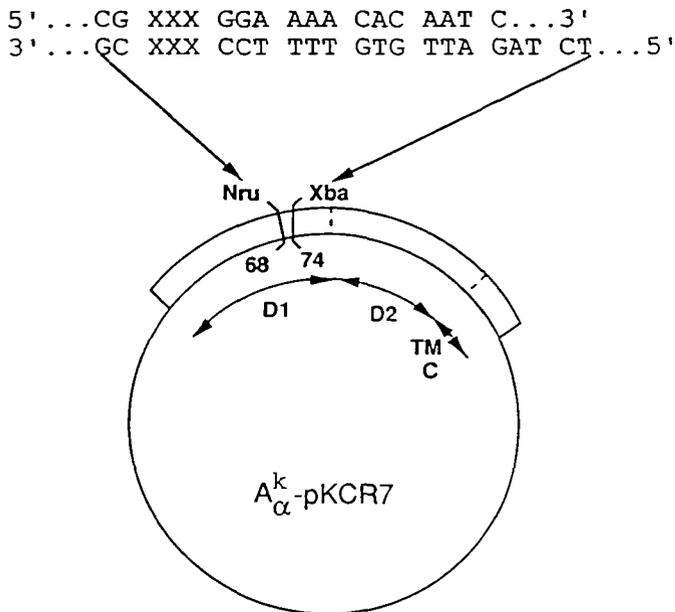


Fig. 1. Mutagenesis strategy. The plasmid A_{α}^k -pKCR7 contains an A_{α}^k cDNA in the expression vector pKCR7 (6,8). The domains of A_{α} are indicated as D1, the membrane-distal domain; D2, the membrane-proximal domain; TM, the transmembrane region; C, the cytoplasmic tail. The cloning vector was prepared by cutting at the (artificial) *Nru*I and *Xba*I sites, leaving a gap at the nucleotides coding for amino acids 68–74. This gap was filled via ligation of the double-stranded oligo mix whose sequence is given and which is described in more detail in the text. XXX marks the positions of the three nucleotides coding for amino acid 69, and represent random incorporation of the four bases at each position.

position 69 of the α chain. The ability of the panel members to present antigenic peptides to diverse HEL-specific and RNase-specific T cell hybridomas was then evaluated.

Slot-machine mutagenesis (8) was used to create a series of mutant A_{α}^k cDNAs. First, two mixtures of complementary oligonucleotides (oligos) were synthesized as indicated in Fig. 1. During the synthesis of each, we permitted random incorporation of the four nucleotides at the target site—the triplet encoding A_{α}^k amino acid 69. Next, the two complementary oligo mixes were annealed and ligated to A_{α}^k -pKCR7 to replace the corresponding stretch of an A_{α}^k cDNA in the pKCR7 expression vector (Fig. 1). The ligation products were transfected into *E. coli*, 80 colonies were selected randomly, small amounts of plasmid DNAs were prepared from each, and the DNAs were sequenced in the vicinity of the target site. A wild-type A_{α}^k cDNA and several position 69 mutants were obtained; the entire first domain of each was then sequenced. These constructs were individually transfected into L cells together with an expressible wild-type A_{β}^k cDNA and a selection marker (8–10). The transfectants displaying surface A^k molecules were electronically sorted using the pan-Ia monoclonal antibody (mAb) 40B, and expanded.

All of the mutant A_{α}^k chains associated with wild-type A_{β}^k and, without exception, the resulting complexes were expressed at the cell surface. Transfectant lines expressing roughly similar surface levels of A^k molecules could easily be obtained, as shown in Fig. 2 for some cases. These qualitative results suggested that none of the A_{α} position 69 substitutions grossly

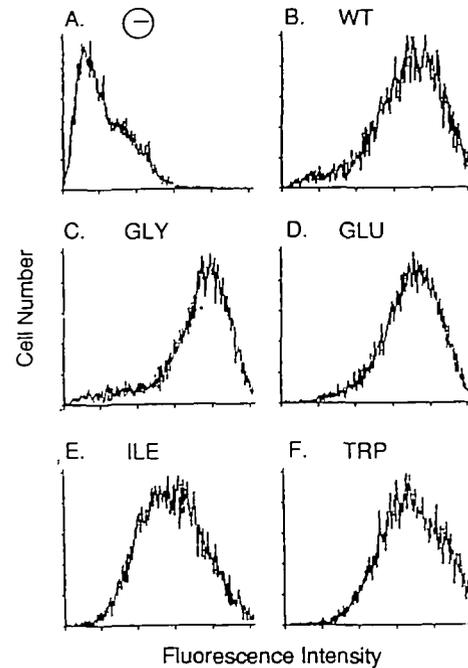


Fig. 2. A^k expression on L cell transfectants. Aliquots of a wild-type and representative mutant transfectant(s) were stained with the anti- A_{β}^k reagent 10 2.16, and were analyzed by cytofluorimetry as described (6,8–10); –, non-transfected control; WT, wild-type A_{α}^k . Gly, Glu, Ile, Trp, amino acids replacing the Thr at position 69 on the A_{α}^k chain. Horizontal axis: fluorescence intensity on a 2-decade log scale.

affect the assembly, transport, cell-surface expression and overall conformation of the A^k complexes—even with such radical amino acid replacements as Glu, Arg, or Trp.

The ability of the mutant lines to present the peptides HEL 46–61 and RNase 41–61 to a panel of T cell hybridomas, whose properties are listed in Table 1, was then assessed. Typical dose–response curves are illustrated in Fig. 3, and the presentation efficiencies derived from such curves are listed in Table 2.

Four points emerge as salient:

(i) All of the mutant A^k complexes were recognized well by at least one of the hybridomas, supporting our contention that the structure of the complex is not grossly perturbed by any of the A_{α} position 69 substitutions. This result also implies that all of the mutant A^k complexes remain capable of binding the peptides tested.

(ii) The amino acid replacements which seemed to have the most profound effect on antigen presentation were those which involve charged or bulky residues, i.e. those at the right-hand side of Table II.

(iii) There was a wide range in the sensitivity of the different T hybridomas to replacements at position 69 of the alpha chain: 3A9 and kLy 11.10 did not recognize any of the mutant A^k complexes, while 2B5.1 responded to all of them, often better than to the wild-type complex.

(iv) As has been discussed elsewhere (7), an Ala replacement at A_{α}^k position 69 provokes an alloresponse from the RNase-specific hybridoma R37. Interestingly, none of the other replacements behaved in a similar fashion.

Table 1. Properties of the T hybridomas

T hybridoma	Source	Immunogen	TCR		Reference
			V _β	V _α	
HEL-specific					
3A9	CBA/J	HEL	8.2	3	13
kLy 11.10	B10.A (4R)	HEL	11	1	14; S. Candeias, unpublished
kLy 4.10	B10.A (4R)	HEL	11		E. Rosloneic, unpublished
1G5	C3H	HEL	6	1	N. Gervois, unpublished
3D3.1	C3H	HEL	8.3	1	N. Gervois, unpublished
1A3.2	C3H	HEL	8.2	4.4	N. Gervois, unpublished
2B5.1	C3H	HEL	8.3	1	N. Gervois, unpublished
RNase-specific					
TS12	CBA/J	RNase	2	1	S. Candeias, unpublished
R37	B10.A (4R)	RNase 41-61	6	4	S. Candeias, unpublished

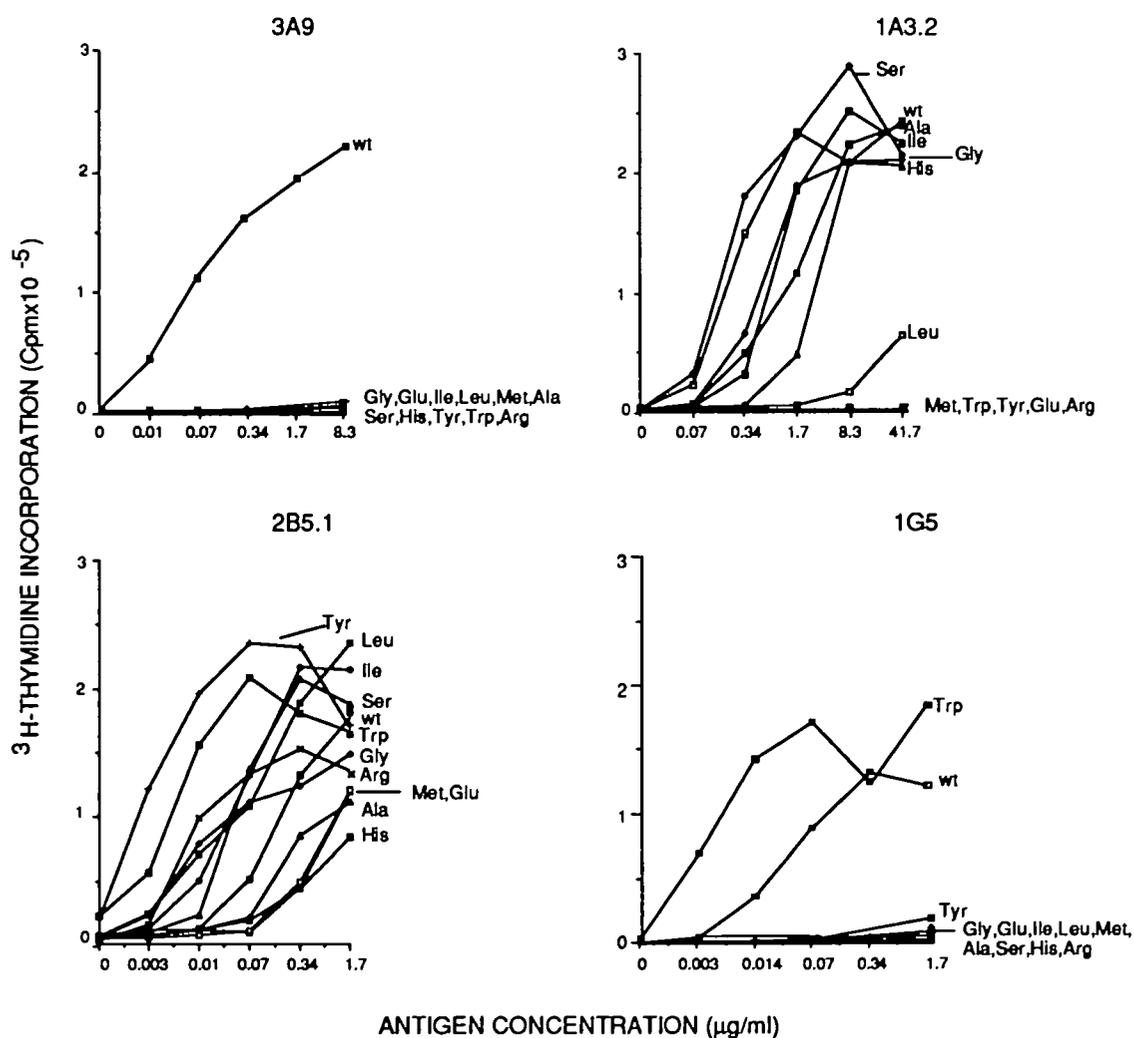


Fig. 3. Dose-response curves. L cells expressing wild-type or mutant A^k molecules were used as antigen presenting cells. The assays were performed in 96-well microtiter plates by incubating 5×10^4 APCs with 5×10^4 T hybridomas and 5-fold dilutions of antigenic peptide in a total volume of 300 μ l. The highest concentration of HEL and RNase peptides was usually 5 μ g/ml. In the negative controls, no antigen was added. After incubation for 24 h, 50 μ l of supernatant was collected and tested for IL-2 content using the CTLL assay, for which 10^4 CTLL cells were used. From the dose-response curves obtained for each mutant L cell line, the antigen presentation efficiency (W/M) was calculated. W represents the antigen concentration required by the wild-type KK cell line to elicit a half-maximum IL-2 response from the T hybridomas, while M indicates the antigen concentration required by the mutant to elicit the same level of IL-2 production.

Table 2. Presentation efficiencies of the mutant APC lines to the various T hybridomas

T hybridoma	Substitution at A _α ^k position 69										
	Gly K69G	Ala K69A	Ser K69S	Ile K69I	Leu K69L	Met K69M	Trp K69W	Tyr K69Y	His K69H	Glu K69E	Arg K69R
HEL-specific											
3A9	–	–	–	–	–	–	–	–	–	–	–
kLy 11 10	–	–	–	–	–	–	–	–	–	–	–
kLy 4.10	–	0.01	0.02	–	–	–	–	–	–	–	–
1G5	–	–	–	–	–	–	2	0.002	–	–	–
3D3 1	0.01	0.02	0.05	–	–	0.01	–	–	–	0.04	–
1A3.2	0.4	0.4	1.5	0.2	0.003	–	–	–	0.02	*	–
2B5.1	1.5	0.4	1.5	2.2	4	0.5	2.0	20	3.0	0.07	2.3
RNase-specific											
TS12	–	–	–	–	0.01	–	–	–	–	–	–
R37	0.7	allo	0.65	–	–	–	–	–	–	–	–

Experiments were performed at several peptide concentrations (with 5-fold dilutions) as well as in the absence of peptide. Presentation efficiencies were calculated as described in Methods. The values are averages of three to four independent experiments, variations between experiments were within a few-fold, at most, of the average shown. Bars indicate undetectable presentation (efficiencies < 0.001). In some experiments, a marginal response was observed and this is indicated by an asterisk. Stimulation of a hybridoma in the absence of peptide is recorded as allo.

What implications do these results have for models of the TCR – MHC molecule – Ag ternary complex?

Previous experiments implicated position 69 on the A_α^k chain as a peptide contact site (6). Here, we have sought to determine just how much structural variation can be tolerated at this position. Our results indicate that diverse amino acids, of radically different chemistry, can be accommodated at position 69 without grossly disturbing the assembly, transport, cell-surface expression or overall conformation of the mutant A^k complexes. Most astonishingly, these diverse amino acid substitutions are also compatible with binding of both peptides tested, since all of the mutant APC lines presented to at least one of the hybridomas. These substitutions include amino acids with the very bulky Trp and Tyr side-chains, which might be expected to virtually fill up the groove locally. On the other hand, T cell recognition is clearly influenced by the various substitutions, but there is tremendous variation in the effect of antigen recognition by the different hybridomas, ranging from exquisite sensitivity to near-total insensitivity.

These observations can be compared with findings from a similar set of experiments on position 75 of the A_α^k chain, a position that is 'up-pointing' according to the model of Brown *et al.*, and thus presumably involved in TCR contacts (8). In this former study, we employed T hybridomas of different antigen specificity (allo), but the results were very comparable to those from the present study: assembly, transport, etc. of the A^k complex were largely unaffected by diverse amino-acid substitutions; T cell reactivity was affected but there was great variety in the individual patterns of recognition. Since position 75 had been predicted to be a T cell receptor contact residue, these findings were precisely what one might have expected. The surprise is that mutations at position 69, a putative peptide contact residue, lead to such similar findings. One would have expected that all T cells specific for a given peptide would either recognize, or not, a particular substitution, according to whether it permitted, or not, peptide binding.

Resolution of this dilemma probably lies in a more sophisticated

view of the TCR – MHC molecule – Ag triplex. For example, there may not be such a clear distinction between TCR – MHC molecule, MHC molecule – Ag and Ag – TCR contacts. It is possible that the MHC molecule moulds the Ag into a particular conformation (6,11) and different features of this molded structure are recognized by different T cells. Following this line of reasoning: contact at position 69 would induce a particular 'nook' or 'cranny' in the tertiary structure of the peptide; some T cells would find this feature totally irrelevant, others would require its exact form, and still others might require its existence but permit some flexibility in its precise form.

Alternatively, it might be that a single peptide is variably disposed within the groove of the class II molecule when participating in different TCR – MHC molecule – Ag triplexes. According to this view, the peptide would fit loosely in the groove—resonating between various positions and/or conformations while making multiple (each non-essential) contacts—until the TCR 'docks on' and locks in a particular disposition. For a given peptide, the allowable positions or conformations could be quite variable, as extreme as register shifts from one end of the groove to the other. Mutations at peptide contact sites on the MHC molecule could prevent, to varying degrees, the different peptide dispositions: position 69 would be critical for recognition by some T cells because it takes part in anchoring the peptide at their recognition site; other T cells would be insensitive to position 69 substitutions because the disposition they recognize does not involve this position. An extreme case might be represented by the T hybridoma 2B5.1—it responds to all of the mutant APC lines and its activation is actually enhanced by the Trp and Tyr substitutions. One could hypothesize that the 2B5.1 recognizes the HEL peptide in a position shifted away from residue 69. By essentially blocking off the groove, the bulky Trp or Tyr side-chains could actually favor this alternative disposition.

The need for a more sophisticated view of T cell recognition was also apparent from the results of the alanine-scan mutagenesis experiments of Peccoud *et al.* (6). Certain 'in-

pointing' residues, including the threonine at position 69, appeared to be direct peptide contacts according to diverse functional criteria; yet competition experiments indicated that alanine replacements at these positions, while drastically affecting T cell recognition, still permitted peptide binding. Similarly, Freed and co-workers showed that mutations at position 69 (Thr → Ile) and 79 (Lys → Glu) in the JE50 B lymphoma line severely affected recognition by most hybridomas but still allowed peptide binding (12). The possibility remained that these surprising and somewhat disturbing findings reflected the particular amino acid replacements—alanine, in particular, being small, uncharged and generally rather innocuous. The present study renders this interpretation inviable.

The two slot-machine mutagenesis experiments (8 and this study) reveal an additional important aspect of the structure of class II molecules. Chemically extreme alterations at positions 69 and 75 do not prevent cell-surface expression of an A^k complex that is capable of binding peptide and of being recognized by at least some T cells. This reveals the great flexibility of the A_α alpha helix and underlines its structural independence from other features of the molecule. Thus, evolution seems to have provided a secondary structure optimally suited to accommodating the polymorphism this chain's function demands.

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Abbreviations

APC	antigen presenting cell
HEL	hen egg lysozyme
oligo	oligonucleotide
RNase	ribonuclease

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