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Defining the Recognition of K^(bm3) and L^d by the Alloreactive 2C T Cell

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Thesis Paper for Research Honors in Biology

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Introduction

The immune system consists (in part) of cells and their products that protect the body from attack by infectious agents. Without this vital system, the body would soon succumb to one or more of these death-causing agents. Fortunately, the immune system eliminates many of these infectious agents before there is any sign of infection. One of the most important groups of cells in this system are the T lymphocytes, or T cells. A specific subset of T cells called cytotoxic T lymphocytes, or CTLs, are responsible for surveying the cell surfaces for foreign substances. Upon recognition of one of these foreign antigens, the T cell becomes activated and kills the cell expressing the antigen. Cells express foreign antigens when infected by foreign microorganisms, (i.e. pathogens) or when they have turned cancerous. The CTL then, is involved in protecting the body from intracellular pathogens and from the development of tumors.

The purpose of this project is to gain a better understanding of allorecognition by T cells through the use of an alloreactive T cell receptor. (In short, allorecognition is the recognition of several molecules by a single T cell receptor.) This understanding will also help us to better understand the positive selection of T cells that occurs during fetal thymic development. During fetal development, immature T cells in the thymus are stimulated to continue development or die, depending upon their ability to recognize class I MHC molecules contained in the thymus (1). Class I MHC molecules are antigen-presenting molecules that are necessary for immune recognition by T cells. The process of positive selection leads to a population of self-MHC-restricted T cells that will recognize peptides only in the context of a class I MHC molecule that was present during development. Occasionally, however, a T cell has the ability to recognize one or more non-self class I MHC molecules that are not normally present in the body. This ability to

recognize one or more non-self MHC molecule(s) that somehow resembles the self MHC molecule is called alloreactivity (2). In some cases, the difference between the training (self) molecule and the allo-molecule (non-self) is a single amino acid substitution, while in other cases, the sequences differ extensively.

Michelle Tallquist, a graduate student at the Mayo Graduate School in Rochester Minnesota, and I worked with the K^{bm3} and L^{d} alleles of the murine class I major histocompatibility complex. These alleles differ at virtually every polymorphic amino acid position within the antigen binding cleft and along the α helices of the MHC molecule (3,4)(Figure 1). The mechanism by which a single T cell receptor (TCR) is able to recognize two or more MHC molecules is not understood. We believe that there is some difference in the orientation of the TCR contact on the MHC/peptide complexes resulting in a change of epitopes (i.e. the portion of the complex recognized) and important contact residues. By selectively mutating several points in the two MHC molecules, we hope to disrupt recognition by altering an important contact residue in one molecule, while not affecting recognition of the other molecule. This result would lead to a better understanding of alloreactivity and positive selection.

Class I MHC Structure

Class I MHC molecules are transmembrane glycoproteins that occur on the surface of all nucleated cells in the body (with the exception of sperm and trophoblast) and play an important role in antigen presentation (1). Each molecule contains a cytoplasmic domain, a transmembrane domain as an anchor, and three extracellular domains (1)(Figure 2). Crystallographic evidence reveals that the important extracellular domains include two membrane-proximal, immunoglobulin-like domains, termed beta-2 microglobulin (β_2M) and alpha 3 (α3), and two

Figure 1:

The L^d and K^{bm3} MHC molecules differ at nearly every polymorphic residue within the antigen binding cleft (3,4). The darkened circles indicate the positions of these amino acid differences within the binding cleft as viewed from above. The numbers indicate the position of the amino acids with respect to the N-terminus (i.e. beginning) of the protein chain. The extreme differences in residues presumably alters the array of peptides bound by the two molecules through alteration of binding pockets as well as steric interference (6).

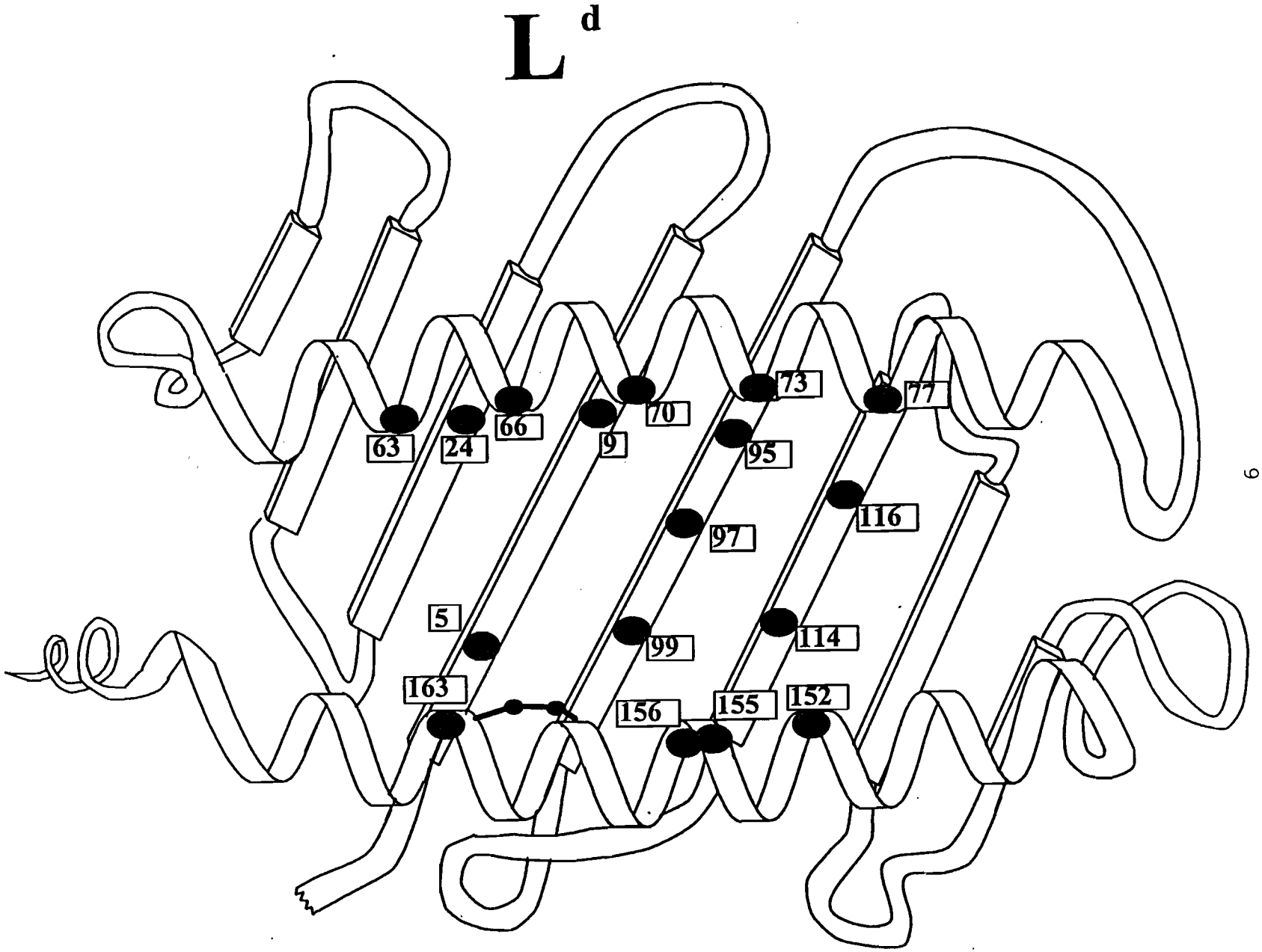

L d
63
24
66
9
70
73
95
77
116
97
5
99
114
163
156
155
152

Figure 2:

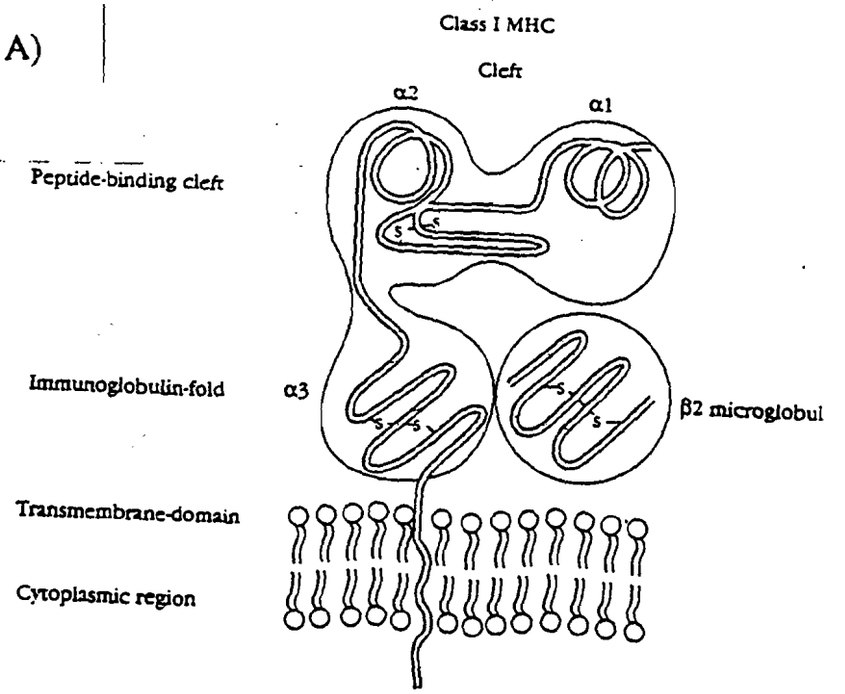
A: Cartoon-type drawing of class I MHC molecule projecting from the cell membrane. The drawing also shows the close association of the β_2-microglobulin molecule which is produced by the transcription and translation of a separate gene.

B: Top view drawing of the antigen binding cleft. The drawing shows the N-terminus of the $\alpha 1$ subunit leading into the antiparallel β sheet, and the direction of the strands within the sheet.

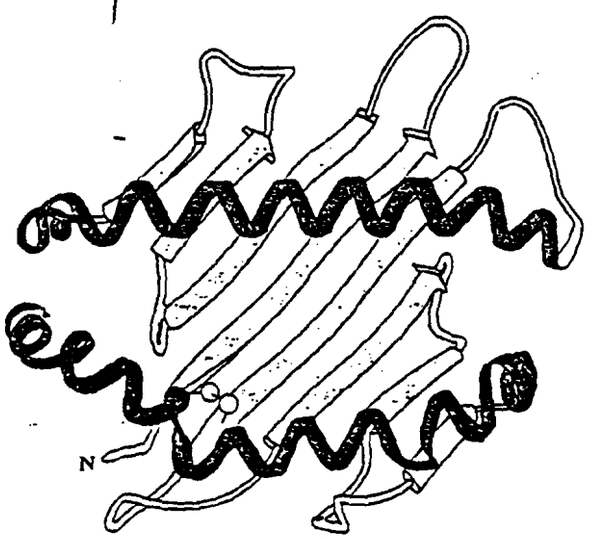
C: Side view drawing of the protein showing the termini of the protein, as well as the direction of each fold. This drawing again shows the β_2-microglobulin in its close proximity to the class I MHC molecule. The binding cleft is also clearly portrayed.

These figures taken from the text book Immunology (1992) by Janis Kuby (1).

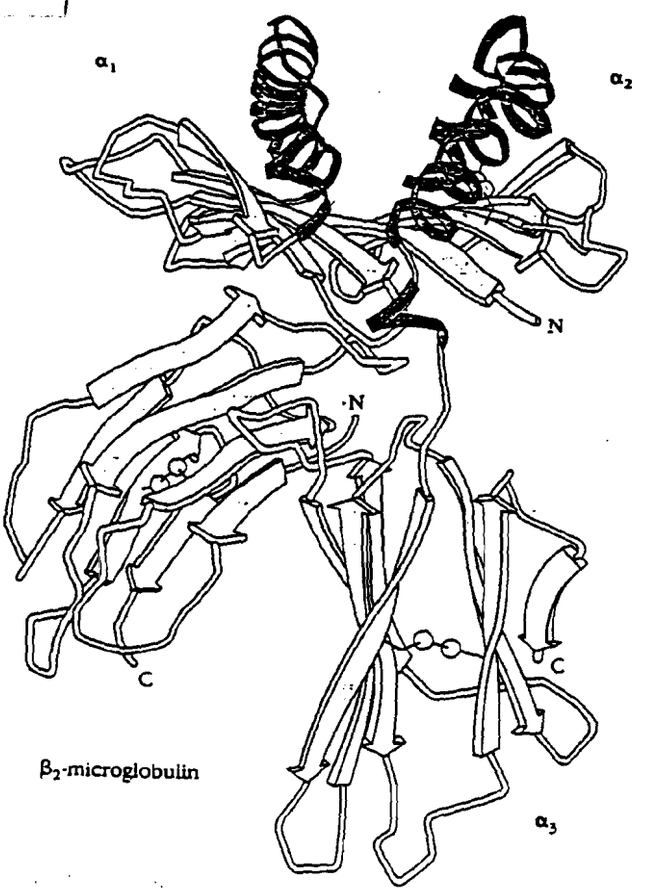
A)


Class I MHC
Cleft
α2
α1
Peptide-binding cleft
Immunoglobulin-fold
α3
β2 microglobul
Transmembrane-domain
Cytoplasmic region

B)


N

C)


α1
α2
N
N
C
C
β2-microglobulin
α3

distal domains, termed alpha 1 and alpha 2 (α1 and α2). The latter domains combine to form eight antiparallel β strands and two long α helices that form the bottom and sides, respectively, of a cleft that binds short peptides (2). The function of the class I MHC molecules is to present endogenously produced peptides on the cell surface within this peptide binding groove or cleft (5). While the α1, α2, and α3 subunits are all encoded by the same set of genes and are one continuous protein, the β_2M subunit is actually an associated protein encoded by a different gene. Association with the β_2M subunit is required for class I MHC expression on the cell surface (1).

Class I MHC Function

MHC/peptide complexes are scanned by $CD8^+$ cytotoxic T cells. Upon recognition of a foreign peptide in association with a class I MHC molecule, $CD8^+$ T cells lyse the infected cell and keep the infection from spreading. This process is especially important in viral diseases and cancers because the peptides produced within a viral-infected or transformed cell differ from those produced in a normal cell.

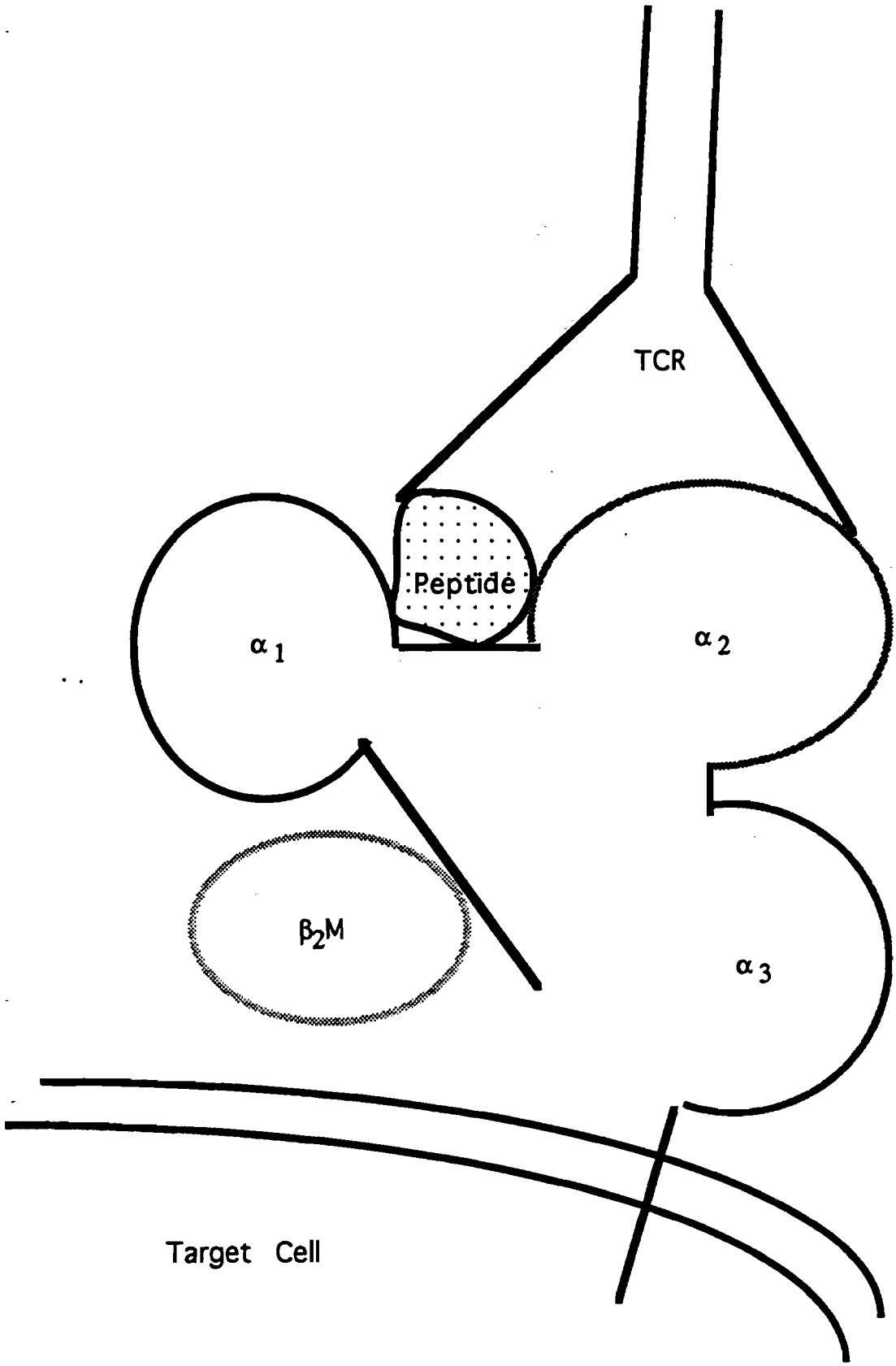
Current dogma holds that there are a number of pockets within the peptide binding cleft that determine the array of peptides which a particular MHC molecule can bind (6). Side chains on the peptide must fit into and interact with these pockets to allow binding of the peptide within the binding cleft through hydrogen bonds and van de Waals interactions (6). The pockets differ between alleles, changing the peptide binding motif of each class I molecule.

T cell recognition occurs when a T cell receptor (TCR) makes contact with and binds to an MHC/peptide complex forming a tri-molecular complex (Figure 3). The TCR is believed to bind to parts of one or both α helices of the MHC molecule, as well as to the exposed side chains of the peptide in the binding cleft (7,8). It has been previously shown that mutations that change

Figure 3:

Representation of the trimolecular complex that forms during immune recognition by a T cell receptor. The TCR binds to contact residues on the α helices and the peptide held within the binding groove. The formation of this trimolecular complex somehow signals the T cell to become activated and lyse the cell.

TCR RECOGNITION OF THE MHC/PEPTIDE COMPLEX



the side chains of the α helices that point up away from the binding cleft have no effect on the peptide array bound by the MHC molecule (9-12). Grandea and Pullen (9,10) have also demonstrated that certain mutations along these side chains lead to an alloresponse by cytotoxic T lymphocytes (CTLs), while others do not change the responsiveness of the CTLs. This leads to our hypothesis that there are certain side chains off the α helices that act as contact residues for the TCR during formation of the trimolecular complex. A study of different TCRs with the same MHC/peptide complex has shown that the contact residues are variable between TCRs (11). We hope to show that a single TCR can have different contact residues depending on the complex it is recognizing. Our hypothesis accounts for the different peptides needed in the clefts of the two molecules to elicit the alloreactive T-cell response because it addresses the complex as a whole.

Methods

All work was done at the Mayo Graduate School in Rochester, Minnesota. The project was accomplished May-August, 1994 and was continued during January, 1995. It was made possible by the summer research program of the Mayo Graduate School, and funding for materials came out of the grants from Dr. Larry R. Pease's lab.

Polymerase Chain Reaction (PCR)

PCR was carried out in a total volume of 100ul containing 0.5ug of sample DNA, 100pmol of each primer oligonucleotide, 10ul of dNTP, 10ul of 10X 15mM Mg^{2+} PCR reaction buffer, 0.5ul of Taq polymerase, and double distilled sterile H_2O (ddH_2O) to volume. Primers were prepared on site in the Mayo molecular biology core facility, and were purified using NAP-10 desalting columns. After purification, they were eluted in ddH_2O and their concentration determined by optical density in a spectrophotometer. The thermal cycler file was set for a total of

25 cycles consisting of a 94°C denaturing step of one minute, a 50°C annealing step of two minutes, and a 72°C extension step of three minutes. In theory, each cycle will double the number of copies of DNA with 100% accuracy. However, Taq polymerase does not have the ability to proofread the strand as it duplicates and is prone to an error frequency of 0.25% (13). This frequency is significantly higher than that observed by Ho et al. (14) in the development of site-directed mutagenesis by overlap extension, which they found to be 0.026%. In either case, because of the possibility of mutations, constructs containing PCR products were carefully checked for sequence fidelity against the known sequence of the molecules.

Genomic L^d DNA was contained in the bacterial expression vector plasmid pBR322, and this was used as our source of L^d template. Oligonucleotide fragments ranging in length from 24 to 36 nucleotides were prepared with specific mismatches that would allow annealing during PCR, but would introduce point mutations during the amplification. These mutations introduced specific restriction sites within the introns before exon 2, between exons 2 and 3, and after exon 3 (Figure 4). K^{bm3} containing the restriction sites had been prepared for previous studies in the lab, so it was not necessary to introduce these mutations.

Splicing by Overlap Extension (SOEing) by PCR

Another process for the introduction of point mutations, SOEing is often used for changing bases in the middle of a stretch of bases (Figure 5). In this case, we wanted to change an amino acid between our new restriction sites. Four primers were required, one at each end of the DNA fragment (in this case, containing restriction sites), and two new ones with complimentary sequences containing a substitution at the same location. Because of their complimentary sequences, one allowed extension from it in the 5' direction, and the other

Figure 4:

Structure of the expression vector 5A7 (3). The darkened boxes represent exons 2 and 3 which code for the $\alpha 1$ and $\alpha 2$ domains, respectively. It is within these two exons that the mutations were made that define each of the constructs.

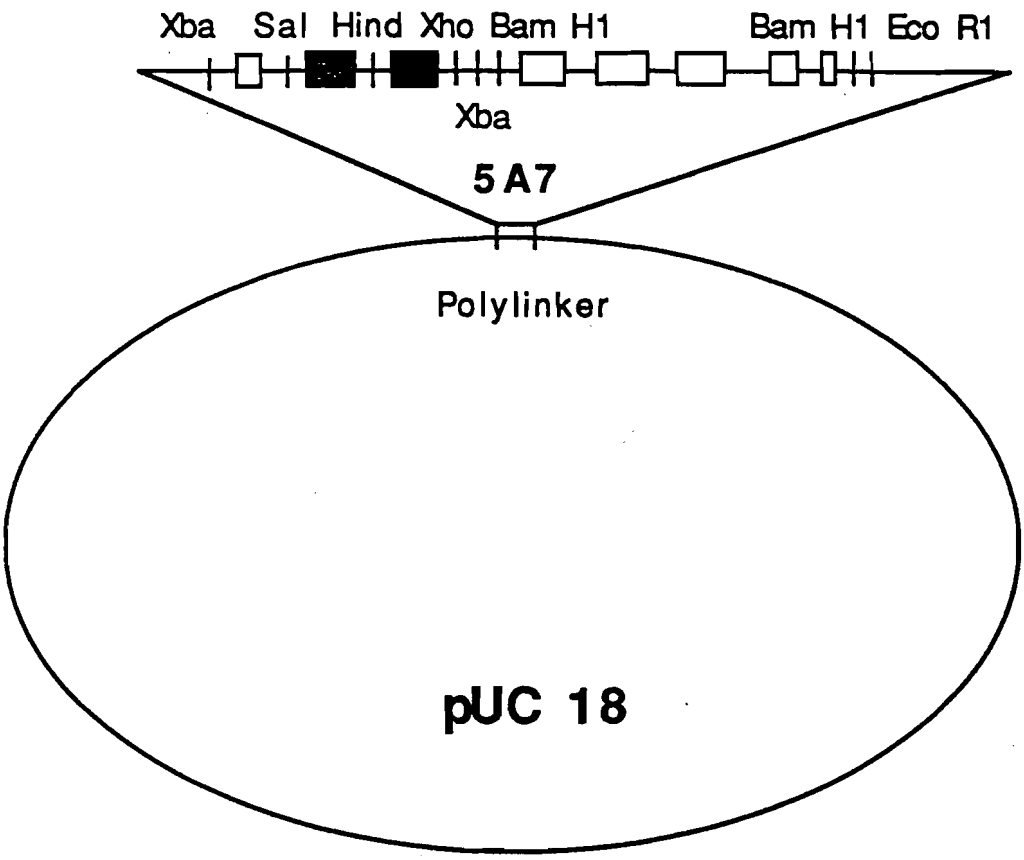
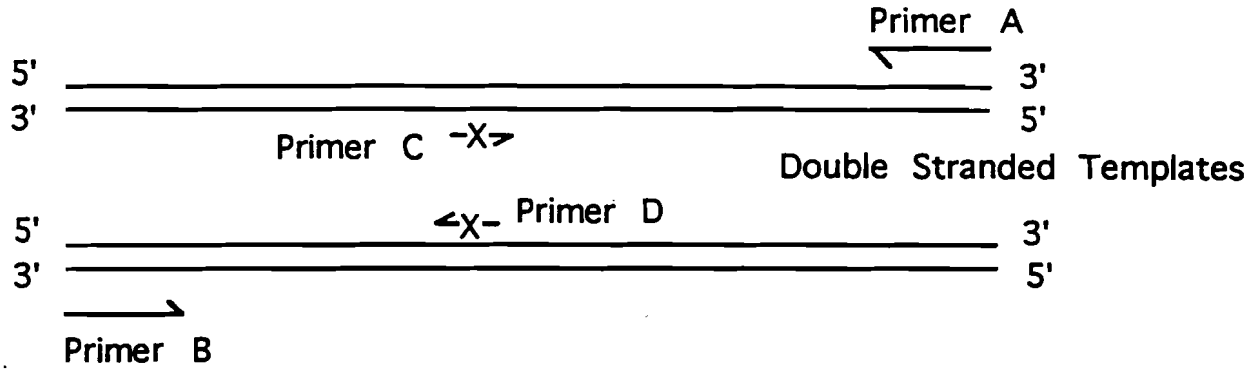

Xba
Sal
Hind
Xho
Bam H1
Bam H1
Eco R1
Xba
5A7
Polylinker
pUC 18

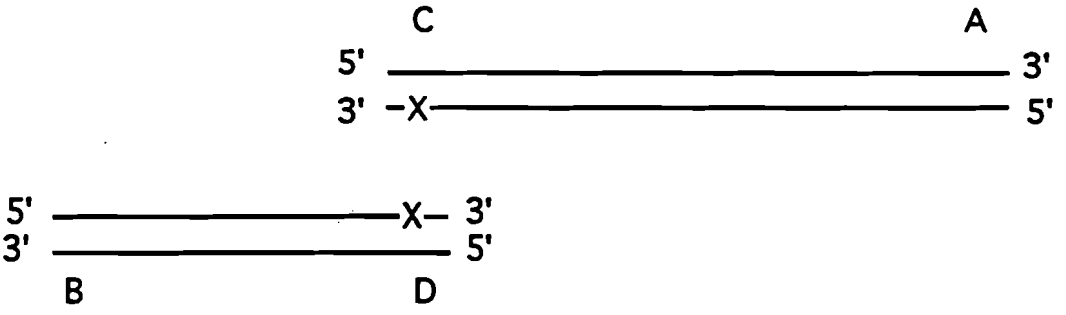
Figure 5:

Explanation of the "SOEing" procedure for site-directed mutagenesis. Four primers are used in a procedure that utilizes PCR twice and results in the precise introduction of nucleotide point mutations.

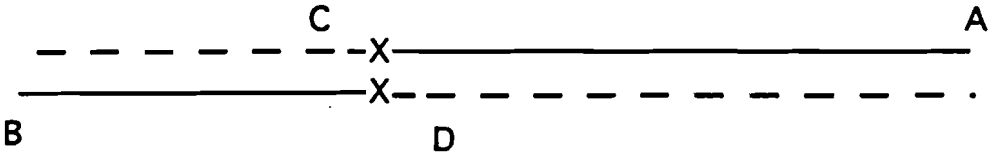
"SOEing" BY PCR



After the first round of PCR, the predominant amplification products are as follows:



These products are complementary in the region surrounding the mutation (X) and can bind to each other during the second round of PCR. The tag ends can then be extended by Taq polymerase.



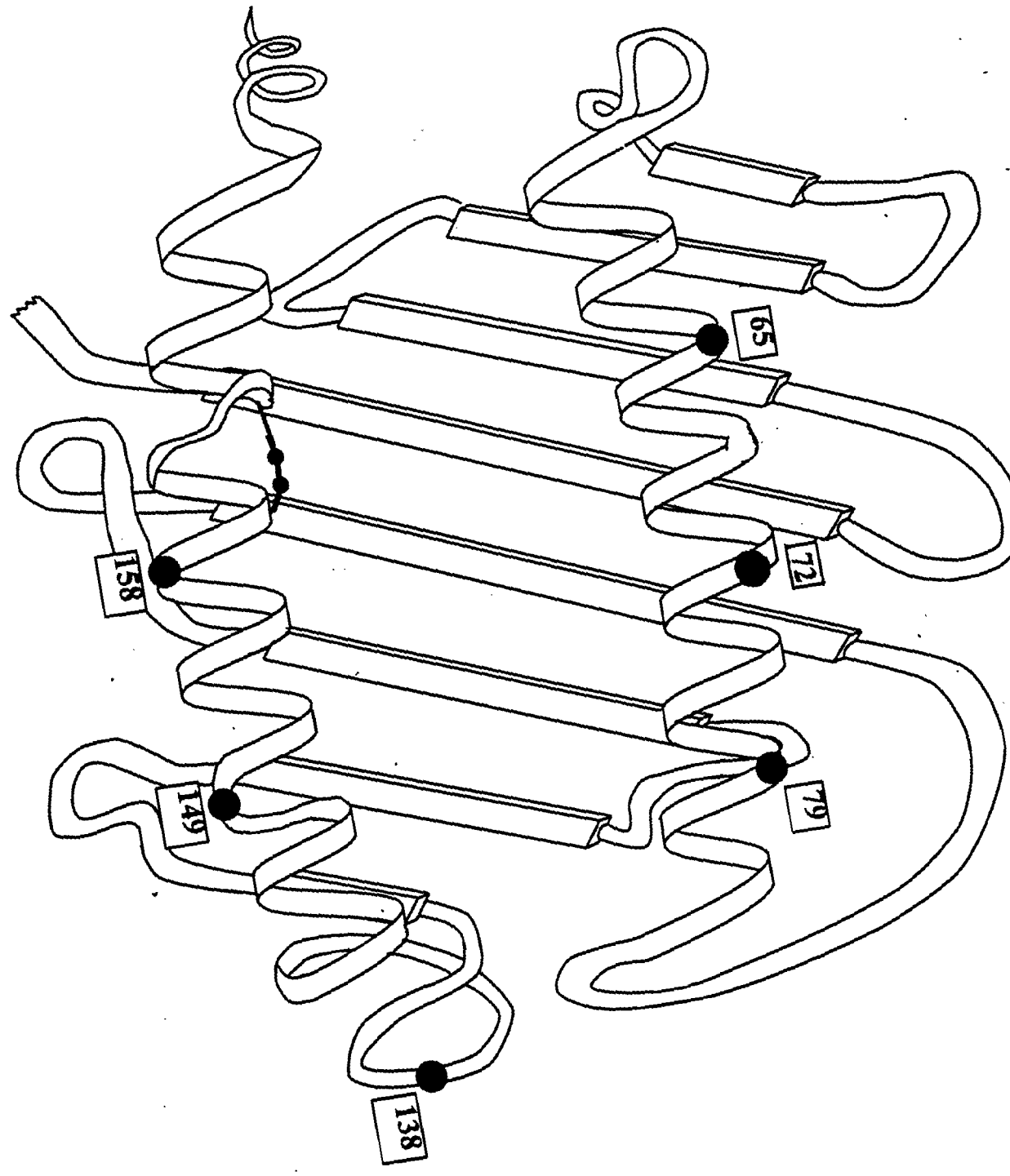
This results in a mutated segment from between points A and B.

terminated extension by annealing farther down the opposite strand. In addition, one of the two previously mentioned restriction primers allowed extension and the other caused termination. In the first step of the SOEing reaction, the 5' restriction primer was paired with the 3' mutation primer, while the 5' mutation primer was paired with the 3' restriction primer. These two reactions were run in separate tubes following standard PCR protocol. A procedure known as Gene Clean (Bio 101) was then run on each of the reaction products. During this procedure, Glassmilk bound the long strands of DNA and held them while excess primers, short strands of DNA (<50 bases), and other reagents were decanted away. The DNA was then recovered by eluting it off of the Glassmilk beads with 55°C water. Gene Clean was also important after restriction digests to eliminate the short end fragments clipped off during the digest, and for recovering DNA from agarose gels after separations.

Following Gene Clean, the products of the first reactions were mixed and a second round of PCR was run using only the two restriction primers. During the heating stage, the two double stranded DNA molecules separated and mixed. Upon cooling at the annealing stage, some of the reannealed molecules were annealed only by their short homologous overlap region (inserted using the mutation primers). Taq polymerase then extended the two ends using the tails as templates. The result was a single DNA molecule that spanned the two restriction sites containing a specifically engineered point mutation. The second PCR step was required to obtain the full stretch of DNA desired. This procedure introduced mutations at amino acids 65, 72, 79, 138, 149, and 158 in both the L^d and the K^{bm3} DNA (Figure 6).

Figure 6:

Positions of the mutated amino acid residues within the peptide binding groove. The darkened circles represent relative positions of changes made using the "SOEing" method. The changes in nucleotides resulted in conservative changes in the amino acid sequence (see Table 1). The changes only alter side chains projecting away from the peptide binding groove and do not affect the array of peptides that bind within the groove (8-12).


65
158
72
149
79
138

Restriction Enzyme Digests

Most cuts were made using 0.5ug of DNA, 10% (1ul) of the appropriate buffer, 10 units (1ul) of the appropriate enzyme, and sterile water to bring the total volume to 10ul. Some digests required a greater quantity of DNA and thus adjustments in the above quantities. Enzymes and buffers were purchased from Boehringer Mannheim, and the reactions were run at the recommended optimum temperature for each enzyme.

Gel Electrophoresis

DNA fragments were separated based on size and charge using 1.5% agarose gels in TBE buffer. TAE buffer was used when the DNA was to be recovered later using Gene Clean. A total volume of 20ul was loaded into each lane of the gel. This volume contained 0.25-0.5ug of DNA and 5ul of ficoll tracking dye in sterile (double deionized) water. A charge of 90 volts for 2 hours was usually sufficient for good separation, but the charge and duration varied depending on the importance of resolution and on time constraints of the day. Ethidium bromide was mixed into the gel before congealing to help visualize the DNA under ultraviolet light.

Transfections

Transfections into BW 5147 cells (thymoma or immortal thymus cells) were accomplished using lipofectamine to create liposomes that could fuse to the cells and release the DNA contained in the liposomes to the cell interior. Lipofectamine is a polycationic lipid that forms complexes with DNA due to ionic interactions with the negatively charged phosphate groups (15). These complexes have a net positive charge, allowing them to associate with and fuse to the charged cell membrane (16). Upon fusion of the outer lipid layer to the plasma membrane, DNA is released to the interior of the cell. The plasmid pRc/CMV was cotransfected along with each of the

constructs as a selective marker. It contained a neomycin resistant gene and allowed selection of successfully transfected cells in a media containing 1mg/ml G418 (synthetic neomycin). Presumably, any cell which integrated the pRc/CMV plasmid also integrated the desired construct because the selection plasmid was added in limited quantities (1ug construct:.5ug selective plasmid). The transfections involved 3.0×10^6 cells which were left in contact with the liposomes for five hours before new media was added. After a 24 hour recovery period, the media was removed and replaced with the selection media containing G418. The cells were then split periodically to keep them from becoming too dense in the flask, and the media was changed every other day to select for the transfected cells.

An alternative transfection procedure was used for our second cell line, the mouse lymphoma cells (L cells) which do not respond well to the above transfection method. Instead, we used a calcium phosphate precipitation procedure to introduce the DNA into the cells. In this procedure, 2M $CaCl_2$ was mixed with the DNA constructs that we wanted to transfect along with a cotransfectant plasmid called pHSV and left to stand for 20 minutes at room temperature while it formed a $CaPO_4$-DNA precipitate. The precipitate was then added to L cells that were plated at a density of $1x10^4/cm^2$. During the 4 hour incubation time, the DNA was endocytosed by the L cells which were then washed free of leftover precipitate and given complete media. After a 48 hour recovery period, the complete media was replaced with HAT selection media.

Cells can synthesize nucleotides by either the de novo or salvage pathways. HAT selection media contains aminopterin which blocks the de novo synthesis of nucleotides, forcing the cell to use the salvage pathway. This salvage pathway requires hypoxanthine and thymidine as well as two enzymes. While the HAT media contains the hypoxanthine and thymidine, L cells are

not capable of utilizing this pathway because of an enzyme deficiency. The transfected cells were separated from untransfected cells because the cotransfected plasmid pHSV contained the genes for the enzymes needed to utilize the salvage pathway. Cells that endocytosed pHSV also presumably endocytosed the plasmid construct. These cells survived in the HAT media and were harvested for analysis.

Cytotoxic T Lymphocyte Assays

CTL assays involved labeling target cells with ^{51}Cr and subjecting them to effector cells. Cells were labeled by adding $Na_2{}^{51}CrO_4$ and incubated for 1 hour, during which time the cells absorbed the radiolabel. Once internalized, the radiolabel was only released in the event of cell death, either by lysis or spontaneous death. Following the labeling, effector cells were added to each well and the mixture was incubated for 5 hours. After incubation, the wells of the plate were harvested and counted in a gamma counter. Spontaneous (background) release of chromium was measured in control wells that did not have effector cells added. Total lysis was determined by the addition of a detergent (Triton X-100) to a set of wells also containing only target cells. Specific lysis was then calculated using the formula:

$$\% \text{ specific lysis} = \frac{(\text{cpm released by CTL} - \text{cpm of spontaneous release})}{(\text{cpm released by detergent} - \text{cpm of spontaneous release})} \times 100$$

Where cpm= counts per minute

We added an additional step when T2 cells were used because they are peptide-deficient and cannot transport MHC molecules with bound peptide to the cell surface. Instead, they have empty peptide binding sites on their MHC molecules. Before adding the effector cells, T2 cells were incubated for one hour with a known peptide to sensitize them. We used a peptide

designated p2Ca on the L^d constructs, and one designated $2CK^{bm3}E$ on the K^{bm3} constructs. Our data suggest these peptides sensitize the respective molecules to 2C TCR recognition (Figure 7).

DNA Sequencing

After the PCR steps, it was necessary to sequence the mutated segment of each construct to make sure the proper mutation was introduced and no new mutations arose. The first step was to transform bacteria with the constructs and culture them overnight. After lysing the bacteria, a plasmid preparation was done and the plasmids were ready for sequencing.

Using oligo primers that bind near the region desired, T7 DNA polymerase synthesized a new strand of DNA until the termination bases were added. Different strands began replication at different times and rates due to reaction kinetics, and ended up being different lengths when the termination base was added. The product was then separated by polyacrylamide gel electrophoresis (PAGE) in a fashion similar to the previously mentioned electrophoresis. One of the bases added to the strands during synthesis was ^{35}S labeled adenine. After drying, the gel was exposed to X-ray film and bands showed up after developing. It was then possible to read the sequence from the gel. This process was tedious and yielded sequences of about 200 bases per primer. Because of the efficiency (350-400 bases per primer) some of the sequencing was done in the automated sequencer at the Mayo molecular biology core facility. The manual sequencing was done to elucidate problems from the automated sequencer such as compressions and unknown bases in the sequence.

Fluorescence Activated Cell Sorting (FACS)

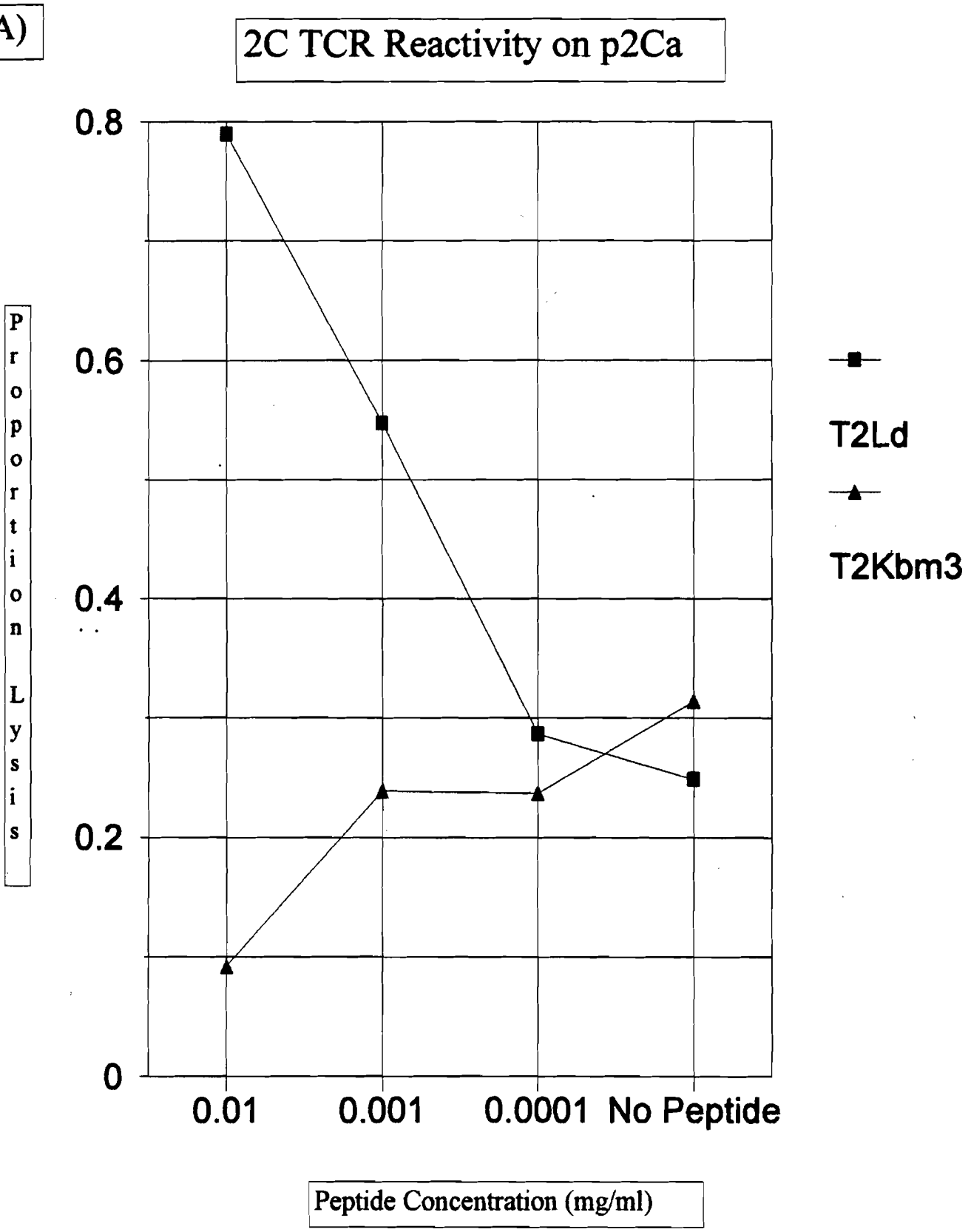
FACS uses antibodies tagged with fluorescent compounds to sort cells based on the presence or absence of fluorescence when struck with laser light. In our sorts, the antibodies

Figure 7:

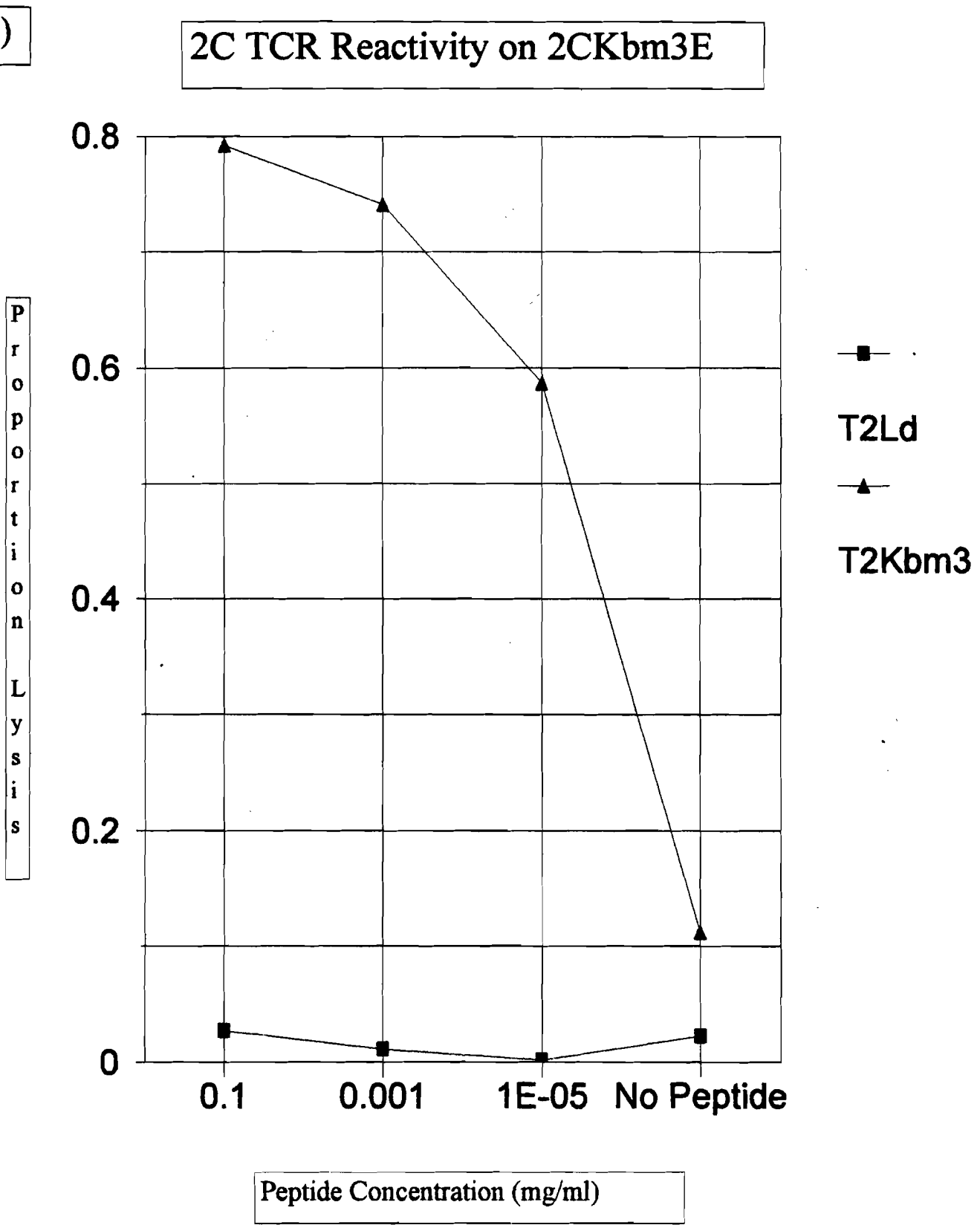
A: Results of the chromium release assay using T2 transport deficient cells and peptide p2Ca. The peptide sensitizes $T2L^d$ cells to 2C TCR recognition in a concentration dependent manner while showing no such sensitization on $T2K^{bm3}$ cells.

B: Results of the chromium release assay using T2 transport deficient cells and the $2CK^{bm3}E$ peptide. This peptide sensitizes $T2K^{bm3}$ cells to 2C TCR recognition in a concentration dependent manner while showing no such sensitization on $T2L^d$ cells.

A)


2C TCR Reactivity on p2Ca
0.8
0.6
0.4
0.2
0
Proportion Lysis
0.01
0.001
0.0001
No Peptide
Peptide Concentration (mg/ml)
T2Ld
T2Kbm3

B)


2C TCR Reactivity on 2CKbm3E
0.8
0.6
0.4
0.2
0
Proportion Lysis
0.1
0.001
1E-05
No Peptide
Peptide Concentration (mg/ml)
T2Ld
T2Kbm3

were specific for the α 3 domain of the MHC class I molecule. We used a primary monoclonal antibody (designated 28-14-8), to bind to the α 3 domain. We then used a secondary antibody produced in a goat against the Fc region of murine antibodies. This secondary antibody was tagged with a compound called FITC that fluoresces in the green spectrum when struck by the laser.

Along with presence or absence of surface molecules, it was also possible to differentiate between live and dead cells, as well as cells of different sizes. These were determined by examining the forward and side scatter of the light. The larger the cell, the more light passes through it to the detector, and the higher its forward scatter. Side scatter is essentially the opposite of forward scatter in that the light that does not pass through the cell is deflected and picked up by another sensor. Granular cells, collapsed cells, and various noncellular particles generally have a high side scatter.

The last compound we used was propidium iodide (PI), which is absorbed by dead cells and fluoresces in the orange spectrum when struck by laser light. Between forward scatter, side scatter, PI, and fluorescence, it was possible to focus on fairly precise populations of cells and sort them out of a larger population.

Materials

5A7 Expression Vector

5A7 is a modified pUC 18 expression vector containing a 1.8-kb XbaI fragment from the murine H-2K^b gene, and a 2.5-kb BamHI fragment from the H-2L^d gene (3)(Figure 4). The XbaI fragment, which encodes the α1 and α2 domains of the class I MHC molecule, has been modified to introduce restriction sites that flank the domains. This allows excision of either domain

independently of the other, which was important in this project. The 2.5-kb BamHI fragment encodes the extracellular α3 domain, the transmembrane domain, and the cytoplasmic domain of the L^d molecule. This was included to allow determination of expression levels of the mutant molecule on the cell surface using the monoclonal (mAb) 28-14-8. The antibody binds to the α3 domain of L^d.

In making the constructs for this project, the SalI and HindIII sites flanking the α1 domain or the HindIII and XhoI sites flanking the α2 domain were used to excise the respective domain so it could be replaced with the desired mutated domain. In the case of the L^d constructs, when one domain was replaced with a mutated domain, the other was replaced by the wild-type L^d domain (i.e. both K^b domains were changed to L^d domains). In the case of the K^{bm3} constructs, the α1 domain of 5A7 had to be replaced because of the mutations at amino acids 77 and 89 that defined the K^{bm3} mutant MHC molecules from the wild-type K^b molecules. The α2 domain could be left unchanged unless a mutation was desired within the domain, as was the case with the 138, 149, and 158 mutants.

Origin of the 2C TCR

The 2C TCR was positively selected in an H-2^b haplotype mouse which had K^b and D^b alleles of the MHC genes. In theory, it should have only responded to these two alleles during antigen recognition (17). However, this did not occur. The 2C TCR was found to be alloreactive on both the K^{bm3} allele (a mutant of the K^b allele) from H-2^b mice, and the L^d allele from H-2^d mice (17). The ability of the TCR to recognize the mutant molecule is not surprising because of the large degree of homology between the molecules that was discussed earlier. The ability to recognize an allele that was not present during selection, however, is quite intriguing. The means

by which this TCR accomplishes this dual recognition is not understood and is the focus of this study.

Cells

The BW cells were purchased from the American Type Culture Collection (Bethesda, MD). 2C T cells were originally derived by Dennis Loh (Washington University) and are now isolated from 2C transgenic mice that are maintained in the Mayo Clinic animal facility. T2 cells were obtained from Peter Crestwell (Yale University).

Results and Discussion

Construct Results

Before we could test the effects of mutations on T cell recognition, it was first necessary to introduce the mutations by using the "SOEing" procedure mentioned above. At the end of the summer we had determined that we had successfully inserted the mutations at amino acid positions 72, 79, 138, 149, and 158 of K^{bm3} and amino acid positions 72, 149, and 158 of L^d. This gave us three pairs of mutants to transfect into L cells (a lymphoma line), in an effort to show differential recognition between K^{bm3} and L^d mutants. Unfortunately, the transfections failed during my absence between August, 1994 and January, 1995. Upon my return in January, we were able to successfully sequence the $K^{bm3}65$ and L^d79 mutants and had four sets of mutants to transfect. Because the first attempt at transfection had failed for an unknown reason, we opted for an alternative cell line that had worked well in the past. We transfected the constructs with mutations at amino acid 72, 79, 149, and 158 into BW5147 cells. The outcome of these transfections is not yet known.

As for the remaining two L^d mutations at amino acid 65 and 138, there were some unforeseen problems. When we sequenced several clones (from different colonies on a streak plate) of the 138 mutant, we found they all shared a single mutation. We hypothesized that this could have been a point mutation arising in an early cycle of PCR and, therefore, showed up in a large proportion of the colonies. This led us to a second attempt at constructing the mutant. Surprisingly, we found the same mutation upon analysis of sequences from the second attempt. Closer analysis of the position of the mutation showed that it was in a position that our manufactured fusmer should have occupied. Although we requested the proper primer sequence, it seemed to have an improper base in it. Either it was constructed improperly, or some biochemical process had mutated the oligo. We were forced to reorder the oligo from the Mayo molecular biology core facility and start over. Unfortunately, it was too late in the month to begin when we received the oligo.

The L^d65 mutant is a bit more complex problem. When we sequenced the mutants, we found that half of the sequence was correct, while the other half (the half of exon 2 that is 5' to our fusmer) was completely wrong. When we entered this nonsense sequence into Genebank, which is a database of gene sequences, it matched it to a region of the *E. coli* genome that codes for glutamine synthetase. It seems that the fusmer is homologous to this region of the *E. coli* genome and can efficiently prime its replication during PCR. PCR requires very small amounts of template DNA for the primers to bind and there seems to be enough genomic *E. coli* template mixed in with the plasmid template to be recognized and amplified during PCR. Because we need to keep the fusmer sequence to introduce the proper mutation, changing primers is not an option. The next step is to obtain a cleaner plasmid preparation. Thus far, most of the plasmid

preparations were done using a kit from Promega (Madison, WI), which gives good plasmid yield and minimal genomic DNA contamination. Unfortunately, this minimal contamination is still too much in this case. In the future, the DNA will have to be purified further using a cesium chloride gradient and ultracentrifugation, which will separate the plasmid DNA from the genomic DNA. Once the separation is complete, the purified plasmid can then be used with the same fusmer in the "SOEing" reaction.

Transfections are Still in Progress

After extensive testing, Kathy Allen, a technician in the Pease lab, determined that the pHSV cotransfectant plasmid was somehow degraded. This explained why our transfection had not survived the selection process at the end of the summer. If the plasmid is degraded, it will not be properly integrated into the host cell DNA and will not be expressed. Without the cotransfected plasmid, the cells will die upon addition of HAT media. Because the selection process is faster and leads to clonal populations of cells expressing the transfected protein, we opted to attempt the L cell transfections again using new pHSV. Preliminary data suggest that the 158 mutants of both alleles are proliferating under selection. The beauty of L cells is that, unlike the BW cells, they are an adherent cell line and will settle individually and begin to divide mitotically to form small colonies. These colonies can then be isolated much like bacterial colonies to give a clonal population. In contrast, BW cells float in the media while dividing and a cell sort must then be done to isolate a majority of the expressing cells. These must then be sorted again, after a few days of proliferation, to yield a population in which the majority of cells express the protein transfected, but which is not clonal. The L cell procedure is quicker and leads to more uniform cultures which is why it was employed in the beginning.

Defining the CTL Assays

Although we were not able to begin the CTL assays on our transfected cells, we were able to run some preliminary tests to determine which of our 2C T cell lines to use, while better defining the system we will be dealing with. There were several 2C T cell lines available in the lab that were characterized and isolated at different times. We found the lines 2C82793 and 2C32494 to be most useful in terms of killing specificity, recovery time, and optimal usage time (i.e. the amount of time needed after stimulation to obtain specific killing). T cell lines must be stimulated weekly with spleen extract to provide them with the targets and cytokines needed for their continued proliferation. The two lines we used gave optimal killing between four and six days after stimulation. Following this period, cells that were not stimulated again were not active enough to kill at an acceptable level. Conversely, using the cells before the four days had elapsed resulted in a high rate of nonspecific target cell lysis due to the hyper-activation of the cells. Other cell lines in the lab required greater periods of time after stimulation before they could be used in an assay, and were deemed unacceptable due to the short time frame of this project.

Determination of Peptides Used

Along with determining the T cell lines to employ, we were also able to support data showing that the 2C TCR recognizes the octapeptide p2Ca in the context of the L^d class I MHC molecule (18). T2 cells have been previously transfected in our lab with both the K^{bm3} and the L^d MHC molecules. These are peptide transport deficient cells that express "empty" class I molecules on their surface. By adding exogenous p2Ca into the empty binding cleft, we were able to show a high degree of specific lysis of the $T2L^d$ cells while observing a low killing rate of the $T2K^{bm3}$ cells (Figure 7A). As a control, we also assayed T2 cells without the addition of

exogenous peptide and, as expected, observed a low specific lysis. We have also shown that the proposed K^{bm3} peptide $2CK^{bm3}E$ is in fact involved in the alloresponse by the 2C TCR. Michelle's thesis work has led her to isolate a potential p2Ca counterpart, $2CK^{bm3}E$, which has produced similar results with $T2K^{bm3}$ cells as p2Ca has produced with $T2K^{d}$ (19)(Figure 7B). These data show that the two MHC molecules are not only very different in their amino acid sequence, but also in the peptide they express (Table 1). This is interesting in terms of defining the different types of alloresponses observed.

Mechanisms of Allorecognition Studied

Allorecognition often involves the recognition of highly different MHC/peptide complexes by the same TCR. However, this is not the only type of alloresponse characterized. It has been shown that alloresponses occur in situations where a single amino acid change has occurred with no affect on the array of peptides bound (9,10). This small change has affected the interaction between the TCR and the MHC/peptide complex leading to an alloresponse. It is also apparent that conformational changes in the MHC molecule or the peptide in the binding groove can cause an alloresponse (10,20), which occurs when a mutation causes a slight perturbation of the complex's shape. There may be several other mechanisms by which alloreactivity occurs, but these are the ones that are well known.

The system employed in this study is of interest because it involves two of the previously mentioned mechanisms. It is obvious that the L^{d} allele is quite different from the 2C training molecule (K^{b}). Therefore, it is believed that a different epitope, but one similar in structure to the positively selecting epitope, may be recognized to allow for the alloresponse. The recognition of a different epitope would require the TCR to encounter the MHC/peptide complex at a different

Table 1. Sequences of oligonucleotide primers and peptides used throughout the project.

Oligonucleotide Sequence	Position	Structural Change
Restriction Primers		
5'GCGTCCTCGGGTCGACCACCGGACCC 3'	Intron 1	SalI site
5'CCCAAGTCCAAGCTTCGGGAACAG 3'	Intron 2	HindIII site
5'GGCTAAGCACTCTCGAGGGAGAAGGCT 3'	Intron 3	XhoI site (K^{bm3})
5'GGCTGGGCCCTCTCGAGGGAGAAGGCT 3'	Intron 3	XhoI site (L^{d})
K^{bm3} Fusmers		
5'GAGCGGGAGACAGACAAAGCCAAGGGC 3'	Amino Acid 65	Gln→Asp
5'AAGGGCAATGAGATGAGTTTCCGAGTG 3'	Amino Acid 72	Gln→Met
5'CGAGTGGACCTGATGACCCTGCTCGGC 3'	Amino Acid 79	Arg→Met
5'ACGGCGGCGGACAAGGCGGCGCTGATC 3'	Amino Acid 138	Met→Lys
5'CACAAGTGGGAGATGGCTGGTGAAGCA 3'	Amino Acid 149	Gln→Met
5'GAGAGACTCAGGACCTACCTGGAGGGC 3'	Amino Acid 158	Ala→Thr
L^{d} Fusmers		
5'GAGCGGATCACGGACATCGCCAAGGGC 3'	Amino Acid 65	Gln→Asp
5'AAGGGCCAGGAGATGTGGTTCCGAGTG 3'	Amino Acid 72	Gln→Met
5'CGAGTGAACCTGATGACCCTGCTCGGC 3'	Amino Acid 79	Arg→Met
5'ACGGCGGCGGACAAGGCGGCGCAGATC 3'	Amino Acid 138	Met→Lys
5'CGCAAGTGGGAGATGGCTGGTGCTGCA 3'	Amino Acid 149	Gln→Met
5'GAGTATTACAGGACCTACCTGGAGGGC 3'	Amino Acid 158	Ala→Thr

Peptides Used

p2Ca: Allows recognition of L^{d} by the 2C TCR.

LSPFPFDL or Leu-Ser-Pro-Phe-Pro-Phe-Asp-Leu

2CK^{bm3}E: Allows recognition of K^{bm3} by the 2C TCR.

EQYKFYSL or Glu-Gln-Tyr-Lys-Phe-Tyr-Ser-Leu

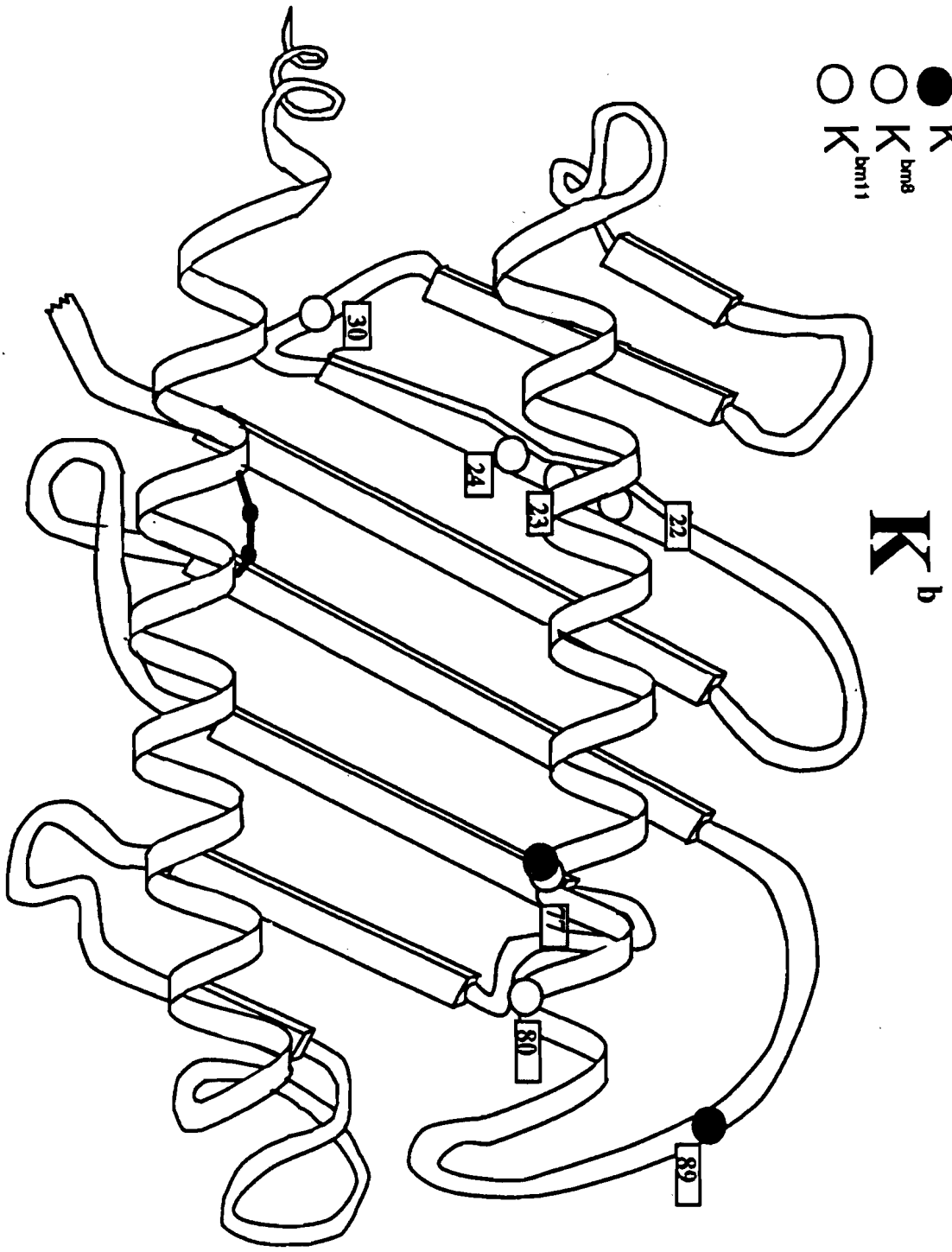
angle or orientation. The training epitope and the alloepitope would then be degenerate recognition sites occurring at different points in their respective complexes. Conversely, the K^{bm3} allele is very similar to the parent K^b molecule and it is believed that the positively selecting epitope is the same epitope that leads to the alloresponse. A slight increase in affinity by the TCR for the MHC molecule would allow allorecognition and CTL activity. This mutant of the K^b molecule differs at two amino acids from the parent molecule, only one of which is associated with the binding cleft (Figure 8). Substitution at position 77 presumably alters the conformation of the molecule and accounts for the increased affinity and alloreactivity by the 2C TCR (3). According to research still in progress (Tallquist, personal communication), it is highly probable that the K^b and K^{bm3} molecules both contain the same peptide recognized by the 2C TCR. This evidence further corroborates the idea that it is the same epitope that is recognized in both molecules. If this is the case, the allorecognition of the K^{bm3} molecule occurs due to the high degree of similarity between the training molecule and the allomolecule, while the L^d alloresponse is directed at the extreme difference of the peptide/MHC complex. This illustrates two different forms of allorecognition. One form is characterized by an MHC/peptide complex that is distinct from self, while the other is defined by a minor structural perturbation that causes a deviation from self that results in allorecognition (19).

Accomplishments of the Project

Once we obtain results from the CTL assays of our transfected mutant molecules, we hope to support our hypothesis of differential epitope recognition. By showing that the epitope for allorecognition differs between the L^d and K^{bm3} molecules, we will show, in an indirect manner,

Figure 8:

Location of the two point mutations that characterize the K^{bm3} mutant molecule. The mutation at amino acid position 77 results in the allorecognition of this molecule by the 2C TCR which was trained on the K^b parent molecule (3).


K bm3
K bm8
K bm11
K b
30
24
23
22
80
89

that the epitopes are also different between the L^d and K^b molecules. This will follow because of the K^{bm3} work done by Pullen et. al. (3).

This project is not of the proper scope to fully address positive selection. However, if our hypothesis is correct, we will further support recent studies (19) implying that the same peptide that positively selects the 2C TCR during development is responsible for a hierarchy of alloresponses in mutant molecules of the K^b allele. This suggests a new strategy for the isolation of molecules that govern positive selection during fetal development. In this strategy, mutants would first be characterized, an allorecognition system determined, and the peptide isolated using HPLC fractionation.

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