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Genetics

Saturation mutagenesis of a major histocompatibility complex protein domain: Identification of a single conserved amino acid important for allorecognition

(*H-2*/cytotoxic T cells)

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ABSTRACT **The interactive association between T lymphocytes and their target cells is an important system of cell–cell interactions. Major histocompatibility complex class I molecules are the cell surface structures recognized by cytolytic T lymphocytes. To define the molecular structures recognized by cytotoxic T lymphocytes, we have saturated the 270-base-pair α1 exon of the *H-2D*^{p} gene with point mutations, rapidly producing a "library" of 2.5×10^3 independent mutants. The library contains enough recombinant clones (each clone encoding approximately one amino acid replacement mutation) to predict a mutation at each nucleotide position of the α1 exon. The functional analysis of the first five transfected gene products tested has shown that mutation of a conserved tyrosine at position 27 to asparagine destroys recognition of the *H-2D*^{p} gene product by polyclonal alloreactive cytotoxic T lymphocytes. Recognition of the same mutant molecule by three monoclonal antibodies and H-2-restricted lymphocytic choriomenengitis virus-specific cytotoxic T lymphocytes is unaffected.**

Mutagenesis has been a useful tool for understanding both gene function and regulation. Some biological systems have allowed the production and selection of a large number of point mutations that approaches saturation of a gene. Classic examples are the definition of the bacteriophage T4 *rII* region (1) and the estimation of the white locus gene size of *Drosophila* (2). The generation of a similar spectrum of mutants in mammalian genes, however, is severely limited by diploidy, genome complexity, and lack of suitable selection schemes. Automated DNA synthesis has allowed the rapid *in vitro* generation of point mutant libraries that contain all the possible single-base substitutions of a small defined "target" sequence (3, 4). We have applied this strategy to the much larger coding sequence target of a major histocompatibility complex (MHC) class I gene for a detailed structure–function analysis. The application of other conventional mutagenesis approaches requires stringent selection to find mutations that occur at a rate of 10^{-4} to 10^{-6}. Our synthetic approach ensures that mutations are recovered at a rate of at least 25% with a controllable frequency per clone. This changes the major obstacle in mutagenesis experiments from the production of mutants to the detailed characterization of systems with mutant phenotypes. We believe the spectrum of mutants created by saturation techniques allows for sampling mutations that would not have been easily recovered by other conventional mutagenesis procedures.

Cytotoxic T lymphocytes (CTLs) recognize epitopes on syngeneic class I molecules when the class I molecule is associated with antigen (5). Peptide fragments may associate with the class I molecule at the cell surface (6), as do MHC class II molecules and antigen (7, 8), and form the basis of recognition by CTLs. CTLs also recognize foreign class I molecules directly (9, 10).

Our laboratory has identified the class I *H-2D*^{p} gene from a genomic library by transfection experiments (11). Sequence analysis revealed a typical murine class I gene (12). Also, we have shown that most of the H-2D^p epitopes recognized by CTLs and B cells reside in the α1 and α2 protein domains (refs. 13 and 14; T. Kanda and J.A.F., unpublished observations), similar to other murine class I molecules (15–17). One difficulty in defining important amino acid residues in the class I molecule is the extreme polymorphism and large size of the class I molecules. This makes site-directed mutagenesis cumbersome, as it is difficult to decide which amino acid to change. We now report the saturation of an entire exon with various point mutations at a defined frequency per clone. The functionally important mutation we describe (tyrosine-27 to asparagine) provides an insight into the recognition of class I molecules by alloreactive and self-restricted CTLs.

MATERIALS AND METHODS

Materials. All restriction endonucleases and DNA ligase were purchased from New England Biolabs.

Bacterial Strains and Plasmids. *Escherichia coli* JM107 was used as a host for phage M13 cloning and propagation of sequencing templates. *E. coli* HB101 was used as a host for all other recombinant DNA work. M13mp19 was used for DNA sequencing.

Synthesis of Mutant Oligonucleotides. Twelve oligonucleotides, which collectively comprise synthetic *H-2D*^{p} α1 exons, were synthesized on an Applied Biosystems (Foster City, CA) DNA synthesizer (3) and purified by polyacrylamide gel electrophoresis. A 1% equimolar solution of all four nucleotide precursors was added to each homogeneous nucleotide precursor reservoir before synthesis. Thus, as synthesis of each oligonucleotide proceeded, an incorrect base was randomly incorporated into the oligonucleotide at a frequency defined by the various concentrations of nucleotide precursors in each reservoir. At each position, there was an equal probability of incorporating an incorrect base, thus generating a point mutation.

Assembly of Mutant Oligonucleotides into α1 Restriction Fragments. Oligonucleotides that had a 5′ internal end, in relation to the final double-stranded structure, were phosphorylated. All 12 oligonucleotides were mixed in equimolar amounts in an Eppendorf tube and placed in 1 liter of boiling water. The water was allowed to cool to room temperature, during which time the oligonucleotides annealed to form

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Abbreviations: MHC, major histocompatibility complex; CTL, cytolytic T lymphocyte; mAb, monoclonal antibody(ies); LCMV, lymphocytic choriomenengitis virus.

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discrete synthetic mutant restriction fragments. This double-stranded DNA was ligated and purified on an 8% acrylamide gel. The ends of the synthetic fragments had been designed to have *Kpn* I and *Sph* I restriction site overhangs at the 5′ and 3′ ends, respectively. The structure of the synthetic mutant exons is shown in Fig. 1. These restriction fragments were ligated into the *Kpn* I and *Sph* I sites of M13mp19, and individual clones were sequenced by the dideoxy method as described (18, 19).

Reconstruction of *H-2D*^{p} Mutant Genes. Each identified α1 mutant sequence has been recloned back into the remainder of the wild-type *H-2D*^{p} gene sequence, replacing the wild-type α1 exon. The plasmid pRM15 contains a genomic copy of the *H-2D*^{p} gene in pUC13. A small portion of intron 3 (*Xba* I–*Bam*HI) was removed during construction. A unique *Kpn* I restriction site was placed 45 bp on the 5′ side of the α1 coding sequence by ligation of a *Kpn* I linker into a *Sma* I site. A unique *Sph* I restriction site was placed 13 bp on the 3′ side of the α1 coding sequence, by ligation of an *Sph* I linker into a *Sma* I site. The α1 exon can be removed from pRM15 by a *Kpn* I–*Sph* I digest. Mutant α1 exons, purified from M13 replicative forms, were then used to reconstruct intact mutant *H-2D*^{p} genes by using standard recombinant DNA techniques.

Transfection of *H-2D*^{p} Mutant Genes into Mouse Ltk$^-$ Cells. Ten micrograms of mutant DNA was linearized with *Eco*RI. One microgram of pMT–113/−85, which carries the thymidine kinase (*tk*) gene under control of the metallothionine promoter (20), was linearized with *Xho* I. The DNA was mixed with 10^7 Ltk$^-$ cells and exposed to an electric field of 2000 V/cm at 25 μF with a time constant of 0.7 msec by using a Bio-Rad Gene Pulser. The mixture was placed on ice for 10 min, and *tk*-positive colonies were selected in HAT medium (with 0.1 mM $ZnSO_4$) as described (11, 20).

RIA of Transfected L Cells. RIA of transfected cells was performed as described (11). The three H-2D^p-specific monoclonal antibodies (mAb; 7-16.10, 135, and 11-20.3) used in this study have been described (21, 22).

CTL Analysis of Transfected Cells. Primary polyclonal alloreactive CTLs were produced from C3H mice as described (23). Spleen cells from C3H mice were cultured for 5 days with irradiated stimulator spleen cells from B10.P mice and assayed by ^{51}Cr release from the L-cell targets. Lymphocytic choriomenengitis virus (LCMV)-specific CTLs were generated *in vivo* by infection of B10.P mice with 10^5 plaque-forming units of Armstrong strain LCMV as described (24) and assayed by ^{51}Cr release on day 6 by using LCMV-infected and uninfected L-cell targets.

RESULTS

Production of the *H-2D*^{p} α1 Mutant Library. To produce a library of genes with point mutations in the α1 exon of *H-2D*^{p}, 12 oligonucleotides were synthesized under unbiased mutagenic conditions. The 12 oligonucleotides were then assembled into synthetic mutant α1 restriction fragments. The mutant α1 fragments were directionally cloned into M13mp19. Individual recombinants from the library of mutants were sequenced. Thus far 51 of 120 recombinant clones sequenced retained the correct α1 reading frame. Thirteen of the 51 clones were wild type, as predicted by a Poisson distribution. Thirty-eight clones contained randomly distributed α1 point mutations. As expected, these mutations result in amino acid substitutions, silent mutations, and an occasional stop codon. Thirty of the 38 clones encode amino acid substitutions that alter the amino acid sequence of the α1 protein domain and are candidates for phenotype analysis. Thus the recovery rate for potentially useful mutations was 25% (30 of 120 mutants), and experiments have shown (R.M. and J.A.F., unpublished observations) that the recovery rate can be much higher. The positions of the amino acid substitutions in these 30 clones are shown in Fig. 2. The 30 mutations described here collectively represent 44 amino acid substitutions in the 90 amino acid α1 protein sequence, a domain known to be critical for CTL recognition. Twenty-five of these positions have been substituted once, 6 positions have been substituted twice, 1 position has been substituted three times, and 1 position has been substituted four times. To visualize the distribution of α1 mutations on the protein structure, we have noted the positions of the amino acids substituted on the α1 and α2 ribbon structure proposed by Bjorkman *et al.* (25) (Fig. 3). We estimate, based on our sequence analysis sampling, that 2500 individual potentially useful α1 mutations exist in the mutant library. Since the α1 coding sequence contains 270 bp and 3 nucleotide substitutions are possible at each position, there are 810 total single-base-change mutants possible (270 positions × 3 substitutions). We expect we have cloned molecules containing at least one mutation at each nucleotide position.

Transfection and Functional Analysis. For clarity, mutants are designated by their clone number (as shown in Fig. 2) followed by the wild-type amino acid residue (in the one-letter amino acid code), the position of the residue, and the mutant amino acid at that position. Thus, D^{p}20(Y27N) is a mutant that changes tyrosine-27 to asparagine. Mutant genes have been transfected, along with the *tk* gene, into mouse Ltk$^-$ cells, and their products have been expressed on the cell surface. Table 1 summarizes the data from five mutant H-2D^p class I molecules. These five mutants have been examined for their ability to (*i*) bind three D^p-specific mAb; (*ii*) be recognized by polyclonal CTLs specific for D^p and LCMV; and (*iii*) be recognized by polyclonal alloreactive CTLs. Four mutants, D^{p}181(D37H), D^{p}164(R14L), D^{p}14-(A11V;E32Q), and D^{p}174(P57Q), express a mutant molecule that we cannot functionally distinguish from a wild-type D^p

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                                                    *
    CCGGGGTCGGCGGGAGGAGGTCGGGGTCTCACCGCGCGCCGCCCCCAGGCCCACACTCGC
CATGGGCCCCAGCCGCCCTCCTCCAGCCCCAGAGTGGCGCGCGGCGGGGGTCCGGGTGTGAGCG

TGAGGTATTTCGTCACCGCCGTGTCCCGGCCCGGCCTCGGGAAGCCCCGGTATATGGAAG
ACTCCATAAAGCAGTGGCGGCACAGGGCCGGGCCGGAGCCCTTCGGGGCCATATACCTTC

TCGGCTACGTGGACAACACGGAGTTCGTGCGCTTCGACAGCGACGCGGAGAATCCGAGGA
AGCCGATGCACCTGTTGTGCCTCAAGCACGCGAAGCTGTCGCTGCGCCTCTTAGGCTCCT

TGAAGCCGCGGGTGCGGTGGATGGAGCAGGAGGGGCCGGAGTATTGGGAGCAGGAGACAC
ACTTCGGCGCCCACGCCACCTACCTCGTCCTCCCCGGCCTCATAACCCTCGTCCTCTGTG

AGAACGCCAAGGACCATGAGCAGAGTTTCCGAGTGAGCCTGAGGAACCTGCTCGGCTACT
TCTTGCGGTTCCTGGTACTCGTCTCAAAGGCTCACTCGGACTCCTTGGACGAGCCGATGA
                *
ACAACCAGAGCAAGGGCGGTGAGTGACCCCGGCATG
TGTTGGTCTCGTTCCCGCCACTCACTGGGGCC
```

FIG. 1. Structure of the synthetic double-stranded mutant α1 exons assembled from 12 oligonucleotides. Coding and noncoding strands were synthesized under unbiased mutagenic conditions and annealed into discrete restriction fragments to form mutant α1 "cassettes." The coding sequence is 270 base pairs (bp) and is shown between the asterisks (*). The underlined and boldfaced nucleotide positions represent the borders of each oligonucleotide. Note the overlap of the coding and noncoding oligonucleotides. The 5′ end retained a *Kpn* I restriction site overhang. The 3′ end retained an *Sph* I restriction site overhang.

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Mutant 1        11        21        31        41        51        61        71        81
Number GPHSLRYFVTAVSRPGLGKPRYMEVGYVDNTEFVRFDSDAENPRMKPRVRWMEQEQPEYWEQETQNAKDHEQSFRVSLRNLLGYYNQSKQ
  DP14 ----------V--------------------Q-----------------------------------------------------------
  DP19 ------------G-------------------------------------------------------------------------------
  DP20 -------------------------N-------------------------------------------------------------------
  DP03 -------------------H-------------------------------------------------------------------------
  DP04 ---------------------------------------------------------------------Y-G---------------------
  DP05 ---------------------------------------------------------------------Q---------------------
  DP23 ------------------------------P------------S-----------------------------------------------
  DP31 ----------G-------------------------------------------------C-------------------------------
  DP33 ---------------------------------------------------------------A---------------------------
  DP35 ---------------------------------------------------------------Q-----------S-------
  DP38 ----------------------------------------------------V-------------------------------------
  DP59 ------------------N-----------------------------------------------------------------------
  DP109---L--C-----------------------------------------------------------------------------------
  DP110-----------------------------------------------------------------------------------------F-----
  DP163-----------------------------------------------------------S-----------------------
  DP164-------------L-----------------------------------------------------------------------
  DP170-----------------------------S-----------------------------------------------------
  DP172-----------------------------------------------------V-----------------------------
  DP174-----------------------------------------------Q-----------------------------------
  DP180-----------------------------------------------------------Q-----------------------
  DP181-----------------------------------H-----------------------------------------------
  DP184-------C-----------------------------------------------------------------------H----
  DP186-----------------------------------H-----------------------------------------N---T--
  DP135-----------------------------------------------------------Y---------------------
  DP136------S-----------------------------------------------------------------------
  DP141-----------------------------------------------V---------------------P------------
  DP153-----------------------------------------------------------------------------H---
  DP154----------------------G-----------------------------------------------QK---------------------
  DP94 ----------------------------D-----------------------------------------------------------
  DP160----------------------------H-------------L---------------------T-----------------------
```

FIG. 2. Deduced amino acid sequences of wild-type and mutant α1 domain of D^p. The wild-type sequence is shown on the top line in the single-letter amino acid code. Mutant clone numbers are shown on the left. A dash indicates where the residue in the synthetic sequence is identical to the wild type. Deduced amino acid substitutions are shown at the appropriate positions. The distribution of mutations is random throughout the target sequence ($\chi^2 = 3.809$; 3 degrees of freedom; $P > 0.7$).

molecule. We therefore present in detail only the data for the analysis of D^p20(Y27N). L cells expressing this mutant molecule fail to be recognized by D^p-specific alloreactive CTLs.

Recognition of Mutant Molecules by D^p-Specific mAb. Fig. 4 shows representative data from RIA analysis of D^p20-(Y27N). Three D^p mAb (7-16.10, 135, and 11-20.3) bind the mutant molecule as well as the wild-type molecule. These antibodies were chosen because they recognize three distinct epitopes on the D^p molecule (ref. 26 and T. Kanda and J.A.F., unpublished observations). All other mutants listed in Table 1 also bind these mAb as well as cells expressing a wild-type D^p molecule. We conclude these mutants show no significant epitope variations detectable with the three mAb used. Also, these data show comparable expression levels between

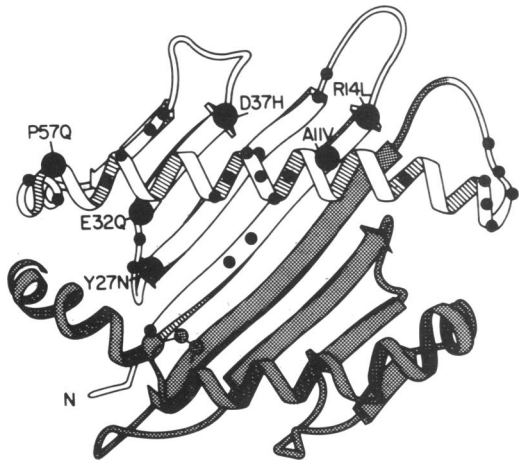

FIG. 3. The positions of the *H-2D*^{p} mutations are displayed on the HLA.A2 ribbon structure redrawn from Bjorkman *et al.* (25). The shaded area represents the α2 structure that was not a target for mutagenesis. The α1 protein structure is shown with large circles marking the amino acid positions that we have functionally analyzed. The distribution of mutations produced by our rapid mutagenesis technique, but not yet functionally characterized, are shown by the small circles.

Table 1. Summary of CTL recognition of mutant D^p molecules

Cell line	mAb A	mAb B	mAb C	H-2-restricted recognition	Allo-recognition
WT	+	+	+	+	+
Ltk$^+$	−	−	−	−	−
D^p14(A11V; E32Q)	+	+	+	+	+
D^p20(Y27N)	+	+	+	+	−
D^p164(R14L)	+	+	+	+	+
D^p181(D37H)	+	+	+	+	+
D^p174(P57Q)	+	+	+	+	NT

Summary of the functional analyses of L cells transfected with five *H-2D*^{p} mutant genes, L cells transfected with the wild-type *H-2D*^{p} gene (WT), and L cells transfected with only the *tk* gene (Ltk$^+$). Reactivity with three mAb (A, B, and C) correspond to those listed in Fig. 4. H-2-restricted recognition of all seven cell types by polyclonal D^p-restricted LCMV-specific CTLs and by polyclonal C3H D^p-allospecific CTL is shown. The wild-type cells are referred to as + for all the D^p-specific reagents (mAb and CTLs). The Ltk$^+$ cells are referred to as − for all the D^p-specific reagents. For the mutants, + indicates that recognition is statistically indistinguishable from the wild-type *D*^{p}-transfected cells (WT), and − indicates that recognition is statistically indistinguishable from the cells transfected with only the *tk* gene (Ltk$^+$). The data for mutant D^p20(Y27N) is provided in detail (see text and Figs. 4 and 5). NT, not tested.

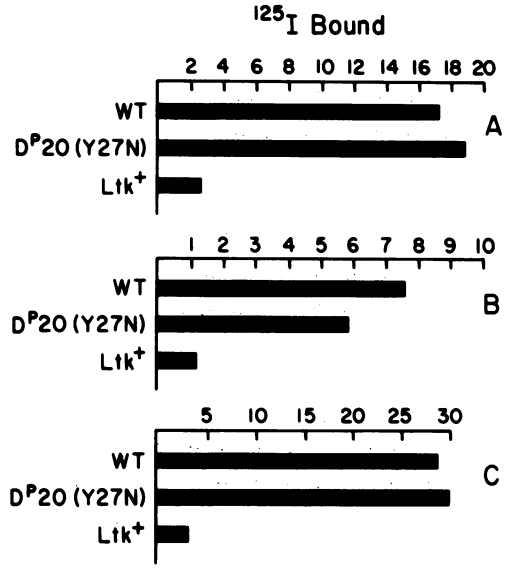

FIG. 4. Cell-surface RIA of L cells transfected with the wild-type *H-2D*^{p} gene (WT), the D^{p}20(Y27N) mutant gene, or the *tk* gene alone (Ltk^{+}). (*A*) Binding with mAb 7-16.10. (*B*) Binding with mAb 135. (*C*) Binding with mAb 11-20.3. All these antibodies have been described (21, 22). Values are the mean value of triplicate samples and are expressed as cpm $\times 10^{-3}$.

wild-type transfected cells and cells expressing mutant molecules. This serves as an important control of antigen density when comparing recognition by CTLs.

Recognition by H-2-Restricted CTLs. We tested spleen cells from LCMV-infected B10.P mice for their ability to lyse LCMV-infected L cells expressing the mutant molecules, wild-type H-2D^{p} molecules, or just the *tk* gene. (B10.P mice express and use the wild-type D^{p} molecule as an LCMV-restriction element). L cells expressing the wild-type D^{p} molecule were lysed, but only if cells were infected with LCMV. The L-cell line expressing only the *tk* gene was not lysed, regardless of LCMV infection. LCMV-infected D^{p}20-(Y27N) targets are killed as efficiently as LCMV-infected cells expressing the wild-type D^{p} molecule (Fig. 5*A*). The other mutants listed in Table 1 were also killed at levels indistinguishable from wild-type transfected cells. If cells were not infected by LCMV, they were not killed (data not shown). Thus, the five mutants express a cell-surface restriction element indistinguishable from the wild-type product by polyclonal CTLs specific for wild-type D^{p} and LCMV.

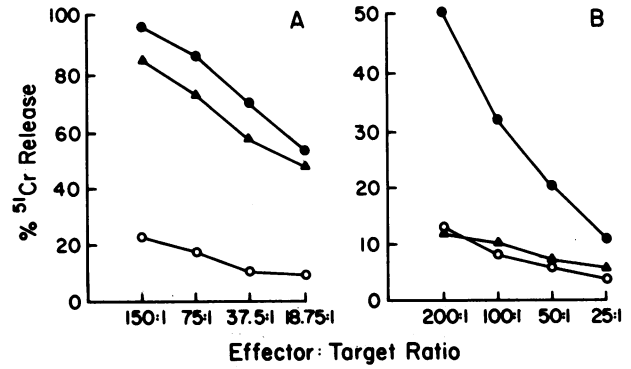

FIG. 5. Killing of L cells transfected with mutant gene D^{p}20-(Y27N). (*A*) Killing of LCMV-infected L cells transfected with the wild-type gene (●), with D^{p}20(Y27N) mutant gene (▲), or with the *tk* gene alone (○). Effectors were prepared from B10.P mice infected with 10^{5} plaque-forming units of Armstrong strain LCMV for 6 days. (*B*) Killing of L cells transfected with the wild-type *H-2D*^{p} gene (●), with the D^{p}20(Y27N) mutant gene (▲), or with the *tk* gene alone (○) by B10.P-specific alloreactive CTLs.

Allospecific Recognition of D^{p} Depends on Tyrosine-27. To examine allorecognition of the mutant molecules, we prepared B10.P-specific CTLs from C3H mice. As seen in Fig. 5*B*, L cells expressing the wild-type molecule were recognized and killed by the C3H effector cells. In contrast, the mutant D^{p}20(Y27N) was not recognized by the same population of allospecific CTLs. L cells transfected with only the *tk* gene were not killed. Thus we conclude that tyrosine-27 is a critical residue for allorecognition of H-2D^{p} by polyclonal D^{p}-specific C3H CTLs. However, the same mutation does not affect self-recognition of LCMV-infected cells. We have repeated this same experiment by using alloreactive C3H CTLs and D^{p}20(Y27N) targets three separate times and obtained identical results.

DISCUSSION

We have shown that the technique of saturation mutagenesis can be applied to the gene for a large intact eukaryotic protein domain. Our experiments have produced a stable library of point mutations not obtainable by conventional mutagenesis methods. This allows us to sample mutations for polymorphic and conserved amino acid residues. Once significant epitopes for CTL recognition are identified, such as tyrosine-27, additional mutations in this region, either from our library or created by site-directed mutagenesis, can further define this structure in greater detail. It is important to note that prior information did not implicate tyrosine-27 as a critical residue and we believe that this emphasizes how the unbiased mutagenesis described can provide unexpected phenotypic information.

We believe the tyrosine-27 to asparagine mutation is particularly interesting. The C3H CTL population, allospecific for wild-type D^{p} that does not recognize D^{p}20(Y27N), is polyclonal. Such populations are likely to contain a variety of CTLs that recognize at least partially different epitopes on the same D^{p} molecule. Therefore, it was not surprising that most of the assays for these mutant molecules were comparable to wild-type levels. If one of several epitopes was altered by a mutation, then recognition could still occur that resulted in target cell lysis. This serves to further emphasize the extreme nature of the D^{p}20(Y27N) mutation that affects at least the majority of the allospecific C3H CTLs. Since D^{p}20(Y27N) is not recognized by alloreactive CTLs and is indistinguishable from L cells not transfected with D^{p}, we believe we have identified a residue critical for allorecognition.

The three-dimensional structure of the HLA.A2 molecule (25, 27) has allowed us to localize the amino acid replacements used in this study. Assuming the overall class I molecular structure is conserved between mouse and human, tyrosine-27 is at the endpoint of the second $\alpha 1$ β strand. This location is on the floor of the putative peptide-binding pocket and forms contacts with amino acids located in the $\alpha 3$ protein domain and with β_2-microglobulin. Preliminary experiments suggest that the mutant molecule is associated with β_2-microglobulin at the cell surface, ruling out the possibility that a grossly altered interaction between the heavy and light chain of this molecule is the cause of the mutant phenotype (J. Alexander, R.M., J.A.F., and P. Cresswell, unpublished observations). We reason that, due to the placement of this residue and its predicted amino acid contacts, we have not directly affected a contact point with the T-cell receptor. The mutation tyrosine-27 to asparagine may have induced a change in the presentation of polymorphic residues or induced a change in the capacity to bind proposed processed self-peptides (28). The interaction with the $\alpha 3$ domain implies a similarity with the $\alpha 3$ mutant described by Potter *et al.* (29) that also disrupts allo CTL recognition. It is useful to compare D^{p}20(Y27N) with K^{bm8}. The K^{bm8} mutant is pre-

sumably the product of a gene conversion event that changes polymorphic residues at positions 22, 23, and 24 on the same β strand as tyrosine-27. The K^{bm8} molecule is, by definition, altered in allorecognition and does in fact present LCMV antigens to CTLs, albeit at a reduced efficiency (30).

The other phenotypically silent mutants characterized here can also be interpreted in light of the HLA.A2 structure. D^p164(R14L) is in a loop outside the obvious contact regions, as is D^p181(D37H). D^p174(P57Q) and D^p14(A11V;E32Q) are more difficult to understand. A11V is a conservative substitution under the α1 α-helix that might easily be silent. However, E32Q, in the same molecule, is a nonconservative substitution at the start of the third β-strand but does not alter recognition at the polyclonal level. The mutation in D^p174-(P57Q) occurs, at first glance, at an interesting position. The proline at residue 57 is predicted to break the α1 α-helix. The substitution at this position has not produced an alteration that we have detected but, due to the position of this residue, it may warrant further investigation.

Tyrosine-27 is conserved in all MHC class I sequences determined to date (human, mouse, and rabbit) (31). It has been suggested that accessory molecules of the T-cell receptor complex are critical in maintaining an efficient interaction with an MHC ligand. Specifically, the conserved nature of CD8 molecules (Lyt-2 in this case) has led to the suggestion that CD8 stabilizes T-cell–target-cell interaction by binding to conserved portions of the MHC recognition structures (32). Therefore, it is possible that we have disrupted a stabilizing interaction of the Lyt-2 molecule and the D^p target structure by the mutation at the conserved tyrosine-27 residue. However, in light of the placement of the tyrosine-27 residue on the class I structure, it is unlikely that we have directly affected an Lyt-2–D^p interaction.

To raise another point related to the mutation at the conserved tyrosine-27 residue, we believe this mutant is a noteworthy example of the usefulness of this mutagenesis technique, as it probably would not have been isolated by other conventional mutagenesis methods. Cells with other class I mutations affecting antibody recognition have been isolated by negative selection by using a specific mAb and complement. These mutations occur in polymorphic portions of the gene (33–35). We have clearly shown that the epitopes for three noncompeting mAb are not disrupted by the mutation in D^p20(Y27N). Therefore, negative selection with our mAb and complement would have excluded the D^p20-(Y27N) mutant. The naturally occurring K^b mutants are thought to have arisen by gene conversion events (30) through changes at polymorphic but not conserved residues. Therefore, mutations in conserved residues, such as tyrosine-27, could not be recovered among the mutants generated *in vivo*. Also, a completely conserved residue would not have been an immediate choice for site-directed mutagenesis, a technique proven effective if a residue is thought to be critical by other criteria (36–38). Thus we believe this mutagenesis technique is exceptionally useful to rapidly produce a wide spectrum of unbiased mutants and is a powerful tool for structure–function analysis and protein designing studies.

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