A SINGLE AMINO ACID SUBSTITUTION IN THE \( \alpha_3 \) DOMAIN OF AN H-2 CLASS I MOLECULE ABROGATES REACTIVITY WITH CTL

By TERRY A. POTTER,* JEFFREY A. BLUESTONE,† AND T. V. RAJAN*‡

From the Departments of *Pathology, and †Microbiology and Immunology, Albert Einstein College of Medicine, New York 10461; and the ¶Transplantation Biology Section, Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Surface molecules encoded by the MHC provide the context for antigen recognition by T lymphocytes (1). The molecular cloning of the genes, as well as genes for the T cell receptor, has provided a means for studying the relationship between T cell function and the specificity for MHC class I or class II molecules. The molecular mapping of determinants on H-2 class I molecules that are recognized by alloreactive cytotoxic T lymphocytes (CTL) was accomplished by the analysis of hybrid H-2 class I molecules constructed by exon shuffling in vitro. Such studies showed that the reactivity of CTL specific for H-2 class I molecules was directed at determinants in the \( \alpha_1 \) and \( \alpha_2 \) domains (2–4). A similar finding was obtained with CTL directed against chemically modified H-2 class I molecules, as well as H-2 class I-restricted antiviral CTL responses (5–7).

Previously, we isolated by immunoselection a somatic cell mutant that expressed a D\(^d\) molecule that failed to express a serologic determinant (34.2.12) that had been mapped to the \( \alpha_3 \) domain, while retaining all of the serologically defined \( \alpha_1 \) and \( \alpha_2 \) determinants (8). In contrast to what had been predicted from the exon-shuffling experiments, cells expressing this mutant D\(^d\) molecule were not killed by anti-D\(^d\)-reactive CTL. It remained possible that, in addition to the change in the \( \alpha_3 \) domain that resulted in the loss of 34.2.12 binding, there were changes in the \( \alpha_1 \) or \( \alpha_2 \) domain of the variant H-2D\(^d\) molecule, and that the \( \alpha_1/\alpha_2 \) changes were responsible for the loss of CTL reactivity. We have now determined that exon 4 of the mutant D\(^d\) gene has undergone a single mutation resulting in a glutamic acid to lysine substitution at residue 227 in the \( \alpha_3 \) domain. Using oligonucleotide-directed mutagenesis, we have reproduced this mutation in the cloned H-2D\(^d\) gene. Cells transfected with this gene are not recognized by alloreactive anti-D\(^d\) CTL. Our studies therefore raise the possibility that, though not identified in the exon-shuffling experiments, residues in the \( \alpha_3 \) domain of H-2 class I molecules contribute to determinants recognized by CTL.

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Materials and Methods

Mice. All strains of mice used in this study were either bred in our own colony (NIH, Bethesda, MD) or purchased from The Jackson Laboratory (Bar Harbor, ME).

Cell Lines. The D⁺ somatic cell variant ACCb 34.2.12⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓
after electroporation, G-418 sulfate antibiotic (Gibco, Grand Island, NY) was added to a final concentration of 1.0 mg/ml. Clones appeared 14–20 d later and were assayed for expression of H-2D^d by microcytotoxicity using the a1/a2 D^d–specific mAb 34.4.21.

Flow Microfluorometric Analysis of H-2 Antigen Expression. For immunofluorescence staining, 10^6 cells in 100 µl of DME/FCS were incubated for 30 min on ice with an optimal amount (10 µl) of anti-H-2D^d mAb. The cells were then washed and incubated on ice for a further 30 min with FITC-conjugated goat anti–mouse immunoglobulin. After staining, the cells were washed, and the fluorescence of 20,000 viable cells was measured on a FACS II (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Generation of Specific CTL Populations. Allogeneic effector cells were generated by in a 5-d primary culture of C57BL/10 spleen cells as previously described (21, 22). In brief, 5 × 10^6 splenic responder cells or 2 × 10^6 purified T cells were mixed with 4 × 10^6 irradiated (3,300 rad) stimulator cells in a final volume of 2 ml of culture media. An exogenous source of IL-2 (lectin-free Con A–induced supernatant) was added to some cultures to a final concentration of 10%. The cultures were incubated for 5–6 d at 37°C in 10% CO_2.

Measurement of CTL Reactivity by ^51Cr Release. Target cells were radiolabelled with ^51Cr by incubating 2–4 × 10^6 cells in 300 µCi of Na^51CrO_4 in 0.5 ml FCS for 1 h. Radiolabelled cells were then washed three times and resuspended at 5 × 10^6 cells/ml. Effector cells were harvested and resuspended at 5 × 10^6 cells/ml. Effector cells and target cells (100 µl) were incubated together in 96-well U-bottom plates (Costar, Cambridge, MA) for 4–5 h at 37°C. Culture supernatants were harvested using the Titertek Supernatant Collection System (Skatron Inc., Sterling, VA). Specific lysis was determined as: percent specific release = 100 × [(experimental release) – (spontaneous release)]/[(maximum release) – (spontaneous release)]. The experimental lysis represents the release of ^51Cr from target cells incubated with effector cells. The spontaneous lysis is given by the release of ^51Cr from target cells in the absence of effector cells. The maximum release is given by the release of ^51Cr released by target cells in the presence of 0.05 N HCl.

Measurement of Stimulation of the IL-2-secreting Hybridoma. The ability of the transfected cells to stimulate IL-2 production by the T cell hybridoma 3DT.52.5.8 was assayed as described by Greenstein et al. (23). In brief, 10^5 3DT.52.5.8 cells were cultured with 10^5 irradiated stimulator cells in a total volume of 1 ml for 24 h at 37°C. The supernatant was harvested and aliquots were assayed for their ability to support the growth of the IL-2-dependent cell line CTLL-1, by measuring the incorporation of [^3H]thymidine during a 6-h pulse with 2 µCi/well [^3H]thymidine.

Results

Identification of the Mutation in the Somatic Cell Variant. The sequence of the a3 domain of the D^d molecule in the ACCb 34.2.12^-4 H-2^b cell line was determined by sequencing of the H-2D^d mRNA using an oligonucleotide that hybridized to H-2D^d mRNA in the transmembrane region. The sequence of the entire a3 domain was determined and compared with the published nucleotide sequence of H-2D^d (24), as well as the sequence determined for the a3 domain of the H-2D^d molecule of the parental cell line. There was only a single nucleotide difference, a G to A transition near the middle of the domain that resulted in a substitution of lysine in the mutant molecule for the wild-type glutamic acid at residue 227 (Fig. 1). The identification of an acid to base substitution is consistent with the observation that the mutant D^d molecule has a more basic isoelectric point (8). It is noteworthy that in all the H-2 class I sequences determined, residue 227 is an aspartic acid, whereas it is glutamic acid in H-2D^d. Therefore, it is most likely that this residue composes part of the determinant recognized by the H-2D^d-specific mAb 34.2.12.
FIGURE 1. Part of the nucleotide sequence of the wild-type (from ACCb H-2b) and the mutant (from ACCb 34.2.12 H-2b-1) Dd gene as determined by RNA sequencing. The sequence of the entire α3 domain of the Dd mRNA from both cell lines was determined, and the only difference with that of the published H-2Dd sequence (24) was at residue 227, as shown.

The sequence alteration determined for the mutant Dd molecule was confirmed by Northern blot analysis using oligonucleotides specific for the parental and mutant sequences. As can be seen in Fig. 2, RNA from both the parental and mutant cell lines hybridized with the oligonucleotide to the H-2Dd transmembrane region that was used as a primer for the RNA sequencing (Fig. 2, lanes 1 and 2). The oligonucleotide specific for the wild-type H-2Dd sequence from residues 224–230 hybridized with RNA from the parental cell line but not with RNA from the mutant cell line (Fig. 2, lanes 3 and 4). Similarly, the oligonucleotide specific for the mutant sequence in this region hybridized with RNA from the mutant cell line but not RNA from the parental cell line (Fig. 2, lanes 5 and 6). The results of these Northern blots therefore confirm the existence of the mutation identified in the RNA sequencing of the mutant Dd molecule.

Having identified the nature of the mutation in ACCb 34.2.12 H-2b-, we reproduced this change and examined its effect in the cloned H-2Dd gene. The H-2Dd gene cloned in pBR327 was obtained from Dr. D. Margulies, and mutagenesis was performed as described in the Materials and Methods as well as Fig. 3. The cell line R8.15 was chosen as the recipient cell for the reconstructed genes for the following technical reasons: (a) it is readily transfecetable by electroporation; (b) it is a good target for measuring specific CTL reactivity and; (c) it does not possess any H-2d-encoded genes (10).

Clones that were resistant to G418 were examined for expression of H-2Dd by
FIGURE 3. Scheme for oligonucleotide-directed mutagenesis of the fourth exon of H-2D\(^d\). The H-2D\(^d\) gene, cloned as an 8 kb Eco RI fragment in pBR327, was obtained from Dr. David Margulies. Plasmid DNA was cut with Hpa I. Kpn I linkers were ligated and the fragment containing exons 4–8 was cloned into M13mp19. Oligonucleotide-directed mutagenesis was performed as described in the Materials and Methods. The fragment containing exons 1–3 of D\(^d\) was cloned in pBR327 by the addition of Kpn I linkers at the Hpa I site and religating with the unique Kpn I site containing H-2D\(^d\) exons 1–3. Not that although a 700 bp Kpn I–Hpa I fragment in intron 3 is lost in this manipulation, it was not necessary for expression of H-2D\(^d\). The black boxes denote the H-2D\(^d\) exons. Restriction enzyme sites: R, Eco RI; K, Kpn I; Hp, Hpa I.

microcytotoxicity. Three cloned cell lines were selected for study: (a) R8.15, the G418-sensitive cell line without any transfected genes; (b) R8.15 D\(^d\) Glu, a G418-resistant clone that was transfected with pSV2-neo containing the H-2D\(^d\) gene with glutamic acid at residue 227 (wild-type D\(^d\)); (c) R8.15 D\(^d\) Lys, a G418-resistant clone that was transfected with pSV2-neo containing the H-2D\(^d\) gene with lysine at residue 227 (mutant D\(^d\)). The expression of the H-2D\(^d\) molecule on these three cell lines was further analyzed by flow microfluorometry on the FACS. As shown in Fig. 4, the anti-D\(^d\) mAbs 34.2.12 and 34.5.8 did not react with R8.15, but did react with R8.15 D\(^d\) Glu. The R8.15 D\(^d\) Lys cell line reacted with the 34.5.8 but not the 34.2.12 antibody and therefore expressed the same serological change as the somatic cell mutant ACCb 34.2.12-4 H-2b-.

Reactivity of Anti-D\(^d\) CTL for the Transfected Cells. Having established that R8.15 D\(^d\) Lys showed the same loss of a serologic determinant as the original somatic cell variant, we examined whether it would serve as a target for anti-D\(^d\) CTL. As shown in Fig. 5, anti-D\(^d\) CTL generated in a primary in vitro response lysed the R8.15 D\(^d\) Glu but not the R8.15 D\(^d\) Lys cells. This finding suggested that the substitution of Lys at residue 227 of the H-2D\(^d\) molecule in R8.15 D\(^d\) Lys reproduced not only the change in serologic determinants, but also the failure of recognition by anti-D\(^d\)-reactive T cells.

Stimulation of IL-2 Production in 3DT.52.5.8 by the Transfected Cells. The T cell hybridoma 3DT.52.5.8 secretes IL-2 when stimulated by a target cell expressing H-2D\(^d\). The ability of the three cell lines to elicit IL-2 secretion in 3DT.52.5.8 was determined. It was found that both R8.15 D\(^d\) Glu as well as R8.15 D\(^d\) Lys stimulated production of IL-2 by 3DT.52.5.8 (Table I). Therefore, although R8.15 D\(^d\) Lys is not lysed by anti-D\(^d\) CTLs, the mutant D\(^d\) molecule still stimulates an anti-H-2D\(^d\)-specific, IL-2-producing T cell hybridoma.
Site-directed mutagenesis and exon shuffling have proved to be useful approaches to the study of structure-function relationships of H-2 class I molecules (2–7, 25, 26). The difficulty in using site-directed mutagenesis to define structure-function relationships for molecules such as H-2 class I that have as many as 270 extracellular amino acids is deciding what residues to alter and what
residues should be introduced. Exon-shuffling experiments (2–7) have established that allele-specific determinants recognized by alloreactive CTL reside in the \( \alpha 1 \) and \( \alpha 2 \) domains. In contrast, recent studies on the recognition of a hybrid MHC class I molecule between the mouse H-2K\( ^{b} \) and the human HLA-A2 genes have demonstrated that replacement of the H-2K\( ^{b} \) \( \alpha 3 \) domain with that of the HLA-A2 gene can abrogate recognition by some anti-H-2K\( ^{b} \) CTL (27 and Marziali and Bluestone, manuscript in preparation). This observation raises the possibility that the \( \alpha 3 \) domain may be involved in CTL recognition. Our approach to the identification of informative structural changes has been to perform immunoselections with mAbs and characterize the functional and structural changes. We have been particularly interested in the \( \alpha 3 \) domain and have isolated somatic cell mutants with structural changes in the \( \alpha 3 \) domain of the H-2L\( ^{d} \) (28) or H-2D\( ^{d} \) (8) molecule. We found that, for the H-2L\( ^{d} \) mutants, there was no detectable effect on T cell reactivity. In contrast, a somatic cell mutant isolated by immunoselection using an mAb to the H-2D\( ^{d} \) \( \alpha 3 \) domain was no longer recognized by anti-D\( ^{d} \)-reactive CTL (8).

In this paper we have sequenced mRNA encoding the mutant D\( ^{d} \) molecule and identified a glutamic acid to lysine change at position 227 in the \( \alpha 3 \) domain. We have reproduced this mutation in vitro by oligonucleotide-directed mutagenesis of a cloned H-2D\( ^{d} \) gene and transfected the mutant gene into a H-213\( ^{d} \) cell line. Cells transfected with this mutant gene have the same characteristics as the original H-2D\( ^{d} \) somatic cell mutant. Cells transfected with this gene do not express the 34.2.12 determinant nor are they recognized by alloreactive D\( ^{d} \) CTL. As the effector CTL cells used were bulk populations generated in primary in vitro responses and not single cloned lines, they most probably represented a polyclonal response, with specificity for several H-2D\( ^{d} \) allele-specific determinants.

A trivial explanation for this apparent abrogation of CTL recognition by the change at residue 227 is that the change in charge destroys the conformation of the D\( ^{d} \) molecule. Arguments against this explanation are: (a) the reactivity of the mutant D\( ^{d} \) molecule with \( \alpha 1/\alpha 2 \) antibodies such as 34.5.8 (Fig. 4) 34.4.21, 34.4.20, 15.1.5, 20.8.6 (data not shown) is indistinguishable from the wild-type D\( ^{d} \) molecule; (b) the mutant molecule is associated with \( \beta 2 \) microglobulin (data not shown); (c) the mutant molecule stimulates the 3DT:52.5.8 hybridoma to secrete IL-2. It should also be noted that between the allelic variations of the \( \alpha 3 \) domains used in the exon-shuffling experiments there are between four to six

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**Table 1**

Stimulation of IL-2 Production by 3DT:52.5.8 in Response to the Transfected Cell Lines

| Cell line | \[^{3}H\]Thymidine uptake (cpm) |
|-----------|---------------------------------|
| R8.15     | 830 ± 96                        |
| R8.15 D\( ^{d} \) Glu | 10,997 ± 402                   |
| R8.15 D\( ^{d} \) Lys | 9,986 ± 752                    |

* Results are expressed as the mean ± SD of six replicate cultures measuring \[^{3}H\]thymidine incorporation into the IL-2-dependent cell line CTLL-1.
differences that involved changes in charged residues, and these apparently have no effect on recognition by CTL.

Our data would at first seem to be in conflict with the exon-shuffling experiments, which suggested that residues in the α3 domain are not recognized by CTL. It is possible to reconcile our findings with those of the exon-shuffling experiments by proposing that the α3 domain expresses a conserved or monomorphic determinant(s) recognized by CTL, and that this determinant is destroyed by the glutamic acid to lysine substitution at residue 227. If such a putative monomorphic or invariant determinant does exist, then replacement of the α3 domain with the α3 domain of other H-2 class I genes or alleles in the exon-shuffling experiments would not have revealed its presence. Of the three external domains, the most likely one to express a conserved determinant would be the α3 domain, since this region has the greatest level of amino acid homology among different H-2 class I genes and alleles. In particular, residues 200–250 are identical in the H-2Kk, Dd, and Ld genes, H-2Kb shows one substitution, and H-2Kd and H-2Dd each have only two substitutions (24, 29–33). There exists much greater allelic variation in the α1 and α2 domains. It may therefore be that selective pressure has not only generated variation in the α1/α2 domains, but also conserved an invariant α3 domain.

Evidence for the existence of conserved determinants on MHC class I and class II molecules has come from blocking experiments using mAbs (34, 35). These experiments suggest that molecules such as Lyt-2 (for class I) and L3T4 (for class II) molecules (36) provide accessory recognition to that of the T cell receptor. This is particularly evident in clones with lower affinity (37). Furthermore, Dembic et al. (38) have recently demonstrated that transfection of the CD8/Lyt-2 gene enhances cytotoxicity by T cells given the T cell receptor α and β chain genes from a donor CTL line.

Our results establish that residue 227 contributes to a determinant recognized by most CTL but not by the IL-2-producing hybridoma 3DT.52.5.8. The 3DT.52.5.8 hybridoma is L3T4+ and Lyt-2− (23), indicating that these molecules are not involved in the recognition of Dd by the hybridoma. The fact that H-2Dd stimulates IL-2 secretion in 3DT.52.5.8, even in the absence of accessory molecules, suggests that the antigen-specific receptor on 3DT.52.5.8 is of high affinity. We therefore propose that the anti-Dd CTL populations that we assayed need to recognize a conserved determinant in the α3 domain as well as the allele-specific determinants in α1/α2. In contrast, the 3DT.52.5.8 hybridoma needs to recognize only the α1/α2 allele-specific determinants and not the conserved determinant in α3. The noninvolvement of the conserved determinant in recognition by 3DT.52.5.8 may be due either to the hybridoma expressing a receptor of very high affinity for the α1/α2 determinants, or alternatively, that the threshold for IL-2 stimulation may be lower than that required for CTL lysis. We are currently extending these studies by generating CTL that have high affinity for their target cell and are not dependent on accessory molecules, such as Lyt-2, for reactivity. If such high-affinity CTL need only to recognize α1/α2 determinants and not the conserved determinant in α3, we predict that such CTL should be able to lyse cells such as R8.15 Dd Lys, in which the conserved determinant has been destroyed.
Summary

We previously described a somatic cell expressing a variant H-2D\textsuperscript{d} molecule that did not serve as a target for alloreactive anti-D\textsuperscript{d} CTL. The mutant cell line had been isolated by its failure to express a serological epitope present on the H-2D\textsuperscript{d} α3 domain. In the present study the α3 domain of the D\textsuperscript{d} molecule of this somatic cell variant was sequenced and a single nucleotide change resulting in a glutamic acid to lysine substitution at residue 227 was identified. This change was reproduced in the cloned H-2D\textsuperscript{d} gene by oligonucleotide-directed mutagenesis. Cells transfected with this mutant gene were not killed by anti-H-2D\textsuperscript{d} CTL. Because previous studies using hybrid H-2 class I molecules had established that the α3 domain does not express allele-specific determinants recognized by CTL, our results raise the possibility that residues in the α3 domain of H-2 class I molecules are critical for CTL recognition and constitute a conserved (or monomorphic) determinant recognized by CTL.

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