Glu227→Lys Substitution in the Acidic Loop of Major Histocompatibility Complex Class I α3 Domain Distinguishes Low Avidity CD8 Coreceptor and Avidity-enhanced CD8 Accessory Functions

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Summary

Cytotoxic T lymphocyte (CTL) activation requires specific T cell receptor (TCR)-class I major histocompatibility complex (MHC) antigen complex interactions as well as the participation of coreceptor or accessory molecules on the surface of CTL. CD8 can serve as a coreceptor in that it binds to the same MHC class I molecules as the TCR to facilitate efficient TCR signaling. In addition, CD8 can be "activated" by TCR stimulation to bind to class I molecules with high avidity, including class I not recognized by the TCR as antigenic complexes (non-antigen [Ag] class I), to augment CTL responses and thus serve an accessory molecule function. A Glu/Asp227→Lys substitution in the class I α3 domain acidic loop abrogates lysis of target cells expressing these mutant molecules by alloreactive CD8-dependent CTL. Lack of response is attributed to the destruction of the CD8 binding site in the α3 domain which is likely to disrupt CD8 coreceptor function. The relative importance of the class I α3 domain acidic loop Glu227 in coreceptor as opposed to accessory functions of CD8 is unclear. To address this issue, we examined CTL adhesion and degranulation in response to immobilized class I–peptide complexes formed in vitro from antigenic peptides and purified class I molecules containing wild-type or Glu227→Lys substituted α3 domains. The α3 domain mutant class I–peptide complexes were bound by CTL and triggered degranulation, however to much lower levels than wild-type class I–peptide complexes. In further experiments, it is directly demonstrated that the α3 domain mutant class I molecules, which lack the Glu227 CD8 binding site, still serve as TCR-activated, avidity-enhanced CD8 accessory ligands. However, mutant class I–peptide Ag complexes failed to effectively serve as CD8 coreceptor ligands to initiate TCR-dependent signals required to induce avidity-enhanced CD8 binding to coimmobilized non-Ag class I molecules. Thus the Glu227→Lys mutation effectively distinguishes CD8 coreceptor and avidity-enhanced CD8 accessory functions.

Cytotoxic T lymphocyte activation is a process that involves multiple cell surface protein interactions with antigen-bearing target cells and a cascade of signal transduction events which lead to proliferation and effector functions (1, 2). T cell receptor interaction with MHC class I molecules bound with antigenic peptides on target cells is responsible for the specificity of CD8+ CTL recognition. So-called coreceptors and accessory molecules on the surface of CTL interact with their respective ligands on target cells and also contribute to T cell activation by increasing the avidity between CTL and targets, amplifying TCR-initiated signals, transducing distinct signals, or a combination of these mechanisms (1, 2).

The CD8 molecule is typically expressed as a disulfide-linked α/β heterodimer on the mature murine CD8+ T cell subset and plays a critical role in CD8-dependent CTL recognition and activation (3). In CTL recognition, CD8 can function as a coreceptor by binding to the same MHC class I molecules as the TCR (3–5), as well as an accessory molecule by binding to any class I molecules, including those which do not interact with the TCR (non-Ag class I) (6–10). The relative importance of CD8 coreceptor or accessory interactions with class I molecules toward T cell activation may depend on the density of specific MHC class I–peptide complexes on the target cells as well as the affinity of the interaction between the MHC–peptide complexes and the
binds MHC class I molecules and facilitates CTL activation. It has been shown by site-directed mutagenesis that the negatively charged loop (residues 222-229) in the highly conserved α3 domain of MHC class I molecules plays an important role in CD8-dependent CTL recognition and activation (4, 13-15). For instance, the Glu227-Lys substitution in the Dα3 domain abrogates the CD8-dependent, alloreactive CTL killing of target cells expressing these mutant molecules (4, 13, 14). The inability of murine CD8-dependent CTL to lyse targets bearing the α3 mutated class I molecules was attributed to the disruption of CD8 interaction with class I (13-15). Consistent with these results, mutational analysis and adhesion assays using CD8α-transfected Chinese hamster ovary cells, demonstrate that the acidic loop in the α3 domain of HLA class I molecules is a binding site for human CD8α/β homodimers (15). The site(s) on the MHC class I molecules that participate in high avidity TCR-activated CD8 binding has not been identified. Whether the α3 domain acidic loop is essential for TCR-activated CD8α/β heterodimer binding to class I is unknown. That mutations in the α2 domain of HLA class I molecules can also interfere with CD8α/β binding suggest the possibility that other sites in addition to the α3 domain on class I may be involved in CD8 interaction(s) (16, 17), perhaps including activated CD8 binding.

Using immobilized purified chimeric class I molecules consisting of Dαα or Kαα and Dαα or Kαα, in which the Dαα or Kαα is either the wild-type or the mutant with a Glu227-Lys substitution, the role of Glu227 in the α3 domain acidic loop of class I in CD8 coreceptor and TCR-activated avidity-enhanced CD8 accessory functions of cloned CTL was assessed. The mutant class I effectively serves as an activated CD8 accessory ligand, indicating that Glu227 is not required for this type of CD8 interaction. In contrast, the purified mutant class I molecules were unable to effectively coengage TCR and CD8 to initiate TCR-dependent activation events.

Materials and Methods

mAbs. A murine hybridoma that secretes an IgG2a mAb recognizing H-2 Dα molecules, B22.249 (18), was a gift from Dr. U. Hammerling (Memorial Sloan-Kettering Cancer Center, New York). The Kβ-specific mAb, Y3 (IgG2a) (19), and Dαα-specific mAbs, 34-5-8 (igG2a) (20) and 34-2-12s (IgG2a) (20) were produced from their respective hybridomas obtained from American Type Culture Collection (ATCC; Rockville, MD). The rat anti-mouse TCRαβ mAb, H57-597 (23), was purchased from PharMingen (San Diego, CA).

Transfected Cell Lines Expressing Chimeric H-2 Class I with Wild-Type or Mutant α3 Domains. M12 (H-2a), a B lymphoma cell line, was transfected with chimeric class I genes as described (13, 24, 25). Exon shuffling genes consisting of the α1 and α2 domain of the H-2 Dα or Kβ genes and either the wild-type or Glu227-Lys substituted α3 domain of the H-2 Dα (13) were transfected into the M12 cell line by electroporation, and are referred to herein as M12.Dα/Dαwt, M12.Dα/DαLys, M12.Kβ/Dβwt, and M12.Kβ/DβLys, respectively.

Cloned CTLs. Clone 3/4 is specific for H-2 Dα and the influenza nucleoprotein (NP) (363-380) of A/PR/8/34 influenza virus and was maintained as described previously (7). The NP peptide used in this study is a 10-mer (NP366-374) with a tyrosine at the NH2 terminus (Y-ASNENMETD) that was synthesized and purified at Multiple Peptide Systems (San Diego, CA). The Kβ-specific CTL clone, 11, was described previously (26, 27).

Purification of Chimeric Murine MHC Class I Molecules Bearing Wild-Type or Mutant α3 Domains. The chimeric H-2 class I molecules, Dα/Dβwt, Dα/DβLys, Kβ/Dβwt, and Kβ/DβLys were purified from 0.5-1.0 X 106 transfected cell lines by immunoadfinity chromatography as described (27, 28) with modifications. B22.249 (Dαα2; 29) and Y3 (Kβα2; 30) mAb columns were used for immunoadfinity purification of Dαα/P and Kβ/Dβ chimeric molecules, respectively. To avoid cross contamination, separate columns were used for the wild-type and the mutant class I molecules. Detergent lysates of the transfected cell lines were passed over the B22.249 or Y3 columns. Columns were washed with 0.1% deoxycholate (DOC), 40 mM NaCl, 10 mM Tris, pH 8.2, and 0.5% DOC, 0.65 M NaCl, 10 mM Tris, pH 8.5. The chimeric molecules were then eluted from the columns using 0.5% DOC, 0.15 M NaCl, 15 mM Na2CO3, pH 10.5 (27). Solid-phase ELISA of purified class I chimeric molecules was performed with various mAbs as described previously (28). Peak fractions showing strong ELISA reactivities were pooled and used as described (27, 28). Protein quantitation was determined by Micro-BCA assay (Pierce Chemical Co., Rockford, IL). Other immunoadfinity-purified H-2 class I mAbs used in this study, including Kβ and Dαα isolated from EL4 cells and Kβ isolated from RDM-4, as well as I-E class II molecules isolated from A20.Cy, were purified as described previously (28, 31).

Assays for CTL Binding and Degranulation. CTL degranulation was assessed by measuring the serum esterase (SE) activity released into the medium with the N'-benzoylxy-carbonyl-L-lysine thio-deryl ester (BLT) assay as described (22). Stimulator cells were pulsed with the NP peptide for 30 min at RT, washed three times with 2% FCS-RPMI, and 3 X 105 stimulator cells were incubated with the BLT assay for 4 h. 20 µl of supernatant was recovered and the OD405 was determined. SE release is calculated as ΔOD405 = OD405 (CTL + stimulators) - OD405 (CTL alone).

CTL adhesion and degranulation responses to purified class I and coimmobilized proteins have been described previously in...
In experiments involving peptide pulsing of purified class I molecules, the class I-bearing wells were incubated with NP peptide resuspended in 2% FCS-PBS at 37°C for 16 h to form peptide–antigen complexes as previously described (7, 32). Either 1 or 2 × 10^5 51Cr-labeled CTL were incubated for 4 h at 37°C on protein-bearing plate wells, and unbound cells were then removed. In experiments using fluid phase anti-TCR-α/β mAb, the antibody was added to CTL in suspension and the cells immediately placed into class I-bearing wells. Cell binding was calculated as percent specific cell bound = 100 × [(cpm bound)/(total cpm - spontaneous cpm)]. Degranulation by CTL was determined simultaneously with CTL binding from the same wells. SE release is expressed as ΔOD_{405nm} = OD_{405nm}(CTL + immobilized class I) − OD_{405nm}(CTL + wells blocked with 2% FCS in PBS). All determinations were done in triplicate for each condition unless specified.

**Cytotoxicity Assay.** Target cells were labeled with ^51^Cr and then pulsed with NP peptide at the concentrations indicated. After washing, 10^5 target cells were incubated with the CD8-dependent, NP-specific CTL clone 3/4 at a 5:1 E/T ratio for 5 h in V-bottom microtiter plates in triplicate. Percent specific ^51^Cr release was calculated as: [(experimental release - spontaneous release)/(maximum release - spontaneous release)] × 100. Results are expressed as mean percent specific ^51^Cr release. In all experiments, the spontaneous ^51^Cr release was <7.5% of the total.

**Anti-CD8 mAb-Blocking Experiments.** For anti-CD8 mAb-blocking experiments, the cloned CTL were incubated with the indicated CD8-specific antibodies at room temperature for 30 min before carrying out the assays. Anti-CD8 mAb, 2.43 was used at a 1:4 dilution of the culture supernatant and purified YTS169.4 was used at 5 μg/ml.

**Results**

**Antigen-specific, CD8^+^ CTL Recognition and Lysis of Target Cells Bearing MHC Class I with Wild-Type or Mutant α3 Domains.** It has been shown that CD8-dependent CTL either failed or had greatly reduced ability, to lyse target cells with amino acid substitutions in residues 222–229 of the α3 domain of MHC class I molecules (4, 13–15, 24, 33). The inability or reduced ability of CD8-dependent CTL to lyse these targets was attributed to failure of CD8 to bind the mutated MHC class I molecules on the target cells. In the present study, we examined the ability of an H-2 D^b^-restricted, influenza NP-specific CTL, clone 3/4, to lyse, or degranulate in response to, target cells transfected with a chimeric cDNA encoding the D^b^-α102 domain and D^a^-α3 domain. The D^a^-α3 domain is either the wild-type or mutant with a Glu227→Lys substitution (13, 24, 25). Both the transfectants, M12.D^b^-D^a^wt or M12.D^b^-D^a^Lys, express comparable levels of the hybrid D^a^ molecules as detected by FACS® analysis using a D^a^-specific mAb, B22.249 (data not shown). The target cells were pulsed with NP peptide at different concentrations, and cytolyis was measured by ^51^Cr release. As expected, and similar to our previous report (24), M12.D^b^-D^a^wt target cells were killed by clone 3/4 to a level comparable to EL4 (H-2^d^), which expresses natural D^a^ molecules (Fig. 1 A). Clone 3/4 also lysed M12.D^b^-D^a^Lys, with two significant differences compared to M12.D^b^-D^a^wt lysis (Fig. 1 A). First, the maximal levels of lysis of M12.D^b^-D^a^Lys (20%) were only one-third to one-half of that of M12.D^b^-D^a^wt (40–50%). Second, the concentration of peptide required to obtain significant lysis was >100-fold higher in M12.D^b^-D^a^Lys than in M12.D^b^-D^a^wt. These results are consistent with our previous report in which lysis by a K^b^-restricted, OVA-specific CTL clone of M12 cells transfected with K^b^-D^a^Lys gene was substantially lower and required higher concentrations of OVA.
peptide than target cells transfected with the K\(^{b}\)/D\(^{b}\)wt gene (24). M12.D\(^{b}\)/D\(^{b}\)wt cell lysis by clone 3/4 CTL was almost completely blocked by CD8\(^{a}\)-specific mAbs 2.43 and YTS169.4 at lower peptide concentrations, while partially inhibited when peptide concentrations were high (Fig. 1 B). This confirmed that the clone 3/4 killing is CD8 dependent, and also showed that CD8 dependency is related to the numbers of MHC class I–peptide complexes involved in the CTL-target interactions (11, 12). Interestingly, even the cytolysis of M12.D\(^{b}\)/D\(^{b}\)Lys was partially blockable by anti-CD8 mAbs (Fig. 1 C) suggesting that CD8 may still participate to some extent in CTL recognition of mutant class I–peptide complexes. Similar to the cytolysis results, M12.D\(^{b}\)/D\(^{b}\)wt cells triggered a substantially stronger SE release than M12.D\(^{b}\)/D\(^{b}\)Lys cells, and these responses were peptide dose dependent (Fig. 2 A). Clone 3/4 degranulation responses triggered by both M12.D\(^{b}\)/D\(^{b}\)wt and M12.D\(^{b}\)/D\(^{b}\)Lys cells were CD8 dependent, as the anti-CD8 mAbs effectively inhibited the degranulation responses (Fig. 2, B and C).

**Table 1. ELISA Reactivity of pD\(^{d}\)/D\(^{d}\)wt and pD\(^{d}\)/D\(^{d}\)Lys**

<table>
<thead>
<tr>
<th>mAbs*</th>
<th>Specificity</th>
<th>pD(^{d})/D(^{d})wt</th>
<th>pD(^{d})/D(^{d})Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>B22.249</td>
<td>D(^{b})(\alpha1)</td>
<td>1.062 ± 0.052*</td>
<td>1.092 ± 0.048</td>
</tr>
<tr>
<td>34-5-8s</td>
<td>D(^{d})(\alpha1)(\alpha2)</td>
<td>0.003 ± 0.001</td>
<td>0.013 ± 0.002</td>
</tr>
<tr>
<td>34-2-12s</td>
<td>D(^{d})(\alpha3)</td>
<td>0.347 ± 0.032</td>
<td>0.009 ± 0.002</td>
</tr>
<tr>
<td>Y3</td>
<td>K(^{b})(\alpha2)</td>
<td>0.005 ± 0.001</td>
<td>0.017 ± 0.001</td>
</tr>
</tbody>
</table>

*ELISA reactivity of pD\(^{d}\)/D\(^{d}\)wt and pD\(^{d}\)/D\(^{d}\)Lys determined with various mAbs.

*First antibody used in solid-phase ELISA assay: purified B22.249 and Y3 were diluted into 2% FCS-PBS and used at 2.5 μg/ml; 34-5-8s and 34-2-12s were used as culture supernatants in 10% FCS-RPML at 1:4 dilution.

*Results were expressed as mean OD\(_{490nm}\) ± SD of triplicate wells.

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CTL cells adhered to immobilized pDβ/Dαwt was more than threefold greater than to pDβ/DαLys (38% compared with 10%). CTL binding to both pDβ/Dαwt and pDβ/DαLys was peptide dose dependent and reached plateau at 10 μM NP peptide, which probably represents a peptide concentration resulting in saturation of peptide binding by the immobilized class I molecules. CTL SE release response showed very similar results (Fig. 3 B), and was also peptide dose dependent with maximal degranulation a minimum of three to five times stronger to pDβ/Dαwt than pDβ/DαLys.

The pDβ/Dαwt and pDβ/DαLys density dependence for CTL binding and degranulation was also investigated (Fig. 4). Both pDβ/Dαwt and pDβ/DαLys were coated separately at several densities and pulsed with a saturating concentration of NP peptide. CTL adhesion (Fig. 4 A) and degranulation response (Fig. 4 B) were density dependent for pDβ/Dαwt and pDβ/DαLys, with pDβ/Dαwt supporting a threefold greater maximal CTL binding (Fig. 4 A) and triggering a fivefold stronger maximal degranulation response (Fig. 4 B) than pDβ/DαLys. When a saturating amount of peptide was used to pulse both the pDβ/Dαwt and pDβ/DαLys at or below saturating densities (0.025 μg/well or less), 8−16-fold more pDβ/DαLys molecules were required to reach the same level of CTL binding to pDβ/Dαwt pulsed with peptide (Fig. 4 A). About a 10-fold higher density of pDβ/DαLys was needed to induce a similar level of clone 3/4 SE release as needed for pDβ/Dαwt, with saturating peptide (Fig. 4 B). An ELISA of immobilized pDβ/Dαwt and pDβ/DαLys done in parallel using Dα-specific mAb, B22.249, confirmed that at the same input of purified class I protein, the immunoreactive Dα epitopes for both pDβ/Dαwt and pDβ/DαLys were at comparable density (Fig. 4 C). Clone 3/4 adhesion (Fig. 5, A and B) and SE release (Fig. 5, C and D) to both NP-pulsed pDβ/Dαwt and pDβ/DαLys were significantly inhibited by anti-CD8 mAb, 2.43 and YTS169.4, suggesting that not only the pDβ/Dαwt, but also the low level pDβ/DαLys-triggered responses are CD8 dependent, thus the pDβ/DαLys may still interact to some extent with CD8 molecules despite the Glu 227→Lys substitution in the α3 domain.

In summary, these results indicate that the Glu 227→Lys mutation in the α3 domain of class I raises the threshold of class I density required for CD8-dependent Ag-specific CTL binding and response severalfold, and substantially lowers the maximal response achieved. This provides the first direct quantitative comparison of the ability of wild-type and α3 mutant class I molecules to serve as Ag-presenting molecules, since it excludes the potential participation of both defined and undefined non-class I accessory molecule interactions.

Both the pDβ/Dαwt and pDβ/DαLys Serve as Effective Ligands for TCR-triggered CD8 Adhesion and CTL Response. CD8 can function both as a "coreceptor" and as an "accessory molecule" during CTL recognition (5−10, 24). Evidence suggests that when TCR is triggered, signals are generated which activate CD8 to a state of higher avidity, and consequently, the activated avidity-enhanced CD8 binds MHC class I molecules and facilitates CTL activation (6−10). For instance, soluble Ab to TCR alone is not a sufficient stimulus for CTL degranulation, however it can trigger avidity-enhanced CD8 accessory-type binding to non-Ag class I molecules despite the Glu 227→Lys substitution in the α3 domain.

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murine class II molecule, I-E<sup>d</sup> (data not shown). We also compared the ability of the pD<sup>b</sup>/D<sup>a</sup>wt and pD<sup>b</sup>/D<sup>a</sup>Lys molecules to provide CD8-dependent costimulatory signals for CTL degranulation in conjunction with soluble anti-TCR antibody. Both pD<sup>b</sup>/D<sup>a</sup>wt and pD<sup>b</sup>/D<sup>a</sup>Lys were found to efficiently activate CTL degranulation response in conjunction with a low concentration of soluble anti-TCR mAb that is otherwise insufficient to stimulate degranulation (Fig. 6 B). The soluble anti-TCR-α/β triggered CTL binding and degranulation to pD<sup>b</sup>/D<sup>a</sup>Lys is comparable to or slightly lower (70-90%) than that of pD<sup>b</sup>/D<sup>a</sup>wt throughout the immobilized class I MHC density curves (Fig. 6, A and B, data not shown). Both binding and SE release of clone 11 in response to either pD<sup>b</sup>/D<sup>a</sup>wt or pD<sup>b</sup>/D<sup>a</sup>Lys non-Ag class I molecules was blockable by an anti-CD8 mAb, 2.43 (Fig. 6, A and B), indicating that the soluble TCR mAb triggered CTL binding to the immobilized class I molecules and observed SE release is mediated by CD8 in an accessory-type interaction. The K<sup>b</sup>/D<sup>a</sup>wt and K<sup>b</sup>/D<sup>a</sup>Lys class I molecules were also purified and tested for their ability to serve as TCR- triggered, avidity-enhanced CD8 ligands for adhesion and response, and yielded similar results to the D<sup>b</sup>/D<sup>a</sup>wt and D<sup>b</sup>/D<sup>a</sup>Lys (data not shown).

We have demonstrated previously that a suboptimal CTL adhesion and response to specific Ag class I can be augmented when non-Ag class I is coimmobilized (7, 27). Augmentation of adhesion and degranulation by the non-Ag class I was shown to be mediated by CD8 in an accessory as opposed to coreceptor capacity (7, 27). We examined whether pD<sup>b</sup>/D<sup>a</sup>wt or pD<sup>b</sup>/D<sup>a</sup>Lys can serve as non-Ag CD8 accessory ligands to enhance CTL binding and degranulation. Both pD<sup>b</sup>/D<sup>a</sup>wt and pD<sup>b</sup>/D<sup>a</sup>Lys were titrated and coimmobilized with a suboptimal density of purified K<sup>b</sup> alloantigen on the wells. The binding and SE release of the K<sup>b</sup> allo-specific clone 11 in response to suboptimal K<sup>b</sup> and coimmobilized pD<sup>b</sup>/D<sup>a</sup>wt or pD<sup>b</sup>/D<sup>a</sup>Lys were determined (Fig. 7). Augmentation of clone 11 binding (Fig. 7 A) and triggering of degranulation (Fig. 7 B) facilitated by the coimmobilized pD<sup>b</sup>/D<sup>a</sup>wt or pD<sup>b</sup>/D<sup>a</sup>Lys was to very similar levels throughout the class I density curves. As expected, the enhanced binding and augmented SE release facilitated by both pD<sup>b</sup>/D<sup>a</sup>wt and pD<sup>b</sup>/D<sup>a</sup>Lys were mediated by CD8, as anti-CD8 mAbs significantly blocked clone 11 binding and response (Fig. 7, A and B). We also found that the coimmobilized pK<sup>b</sup>/D<sup>a</sup>wt or pK<sup>b</sup>/D<sup>a</sup>Lys molecules are equally effective as avidity-enhanced CD8 ligands to enhance CTL binding and degranulation when the D<sup>b</sup>-restricted, NP-specific CTL clone 3/4 is triggered by suboptimal D<sup>b</sup>-NP peptide complexes (data not shown). Taken together, these results show that the Glu227→Lys substitution in the α3 domain of class I molecules does not abrogate the ability of class I to serve as a ligand for activated CD8 accessory interactions. This indicates that the Glu227 in murine class I molecules is not required for TCR-triggered CD8 binding. Since the class I α3 domain mutants can still effectively serve as avidity-enhanced CD8 ligands, the poor ability of
The α3 Glu227→Lys Substituted H-2 Class I-Peptide Complexes Are Defective in Initiating TCR Signals for Avidity-enhanced CD8 Binding to Non-Ag Class I and CTL Response. Since the Dβ/DαLys molecules can serve as effective non-Ag class I ligands for TCR-activated, avidity-enhanced CD8 (Figs. 6 and 7), we investigated whether Dβ/DαLys-peptide complexes are defective in initiating TCR signals for avidity-enhanced, CD8-dependent CTL adhesion and response. We and others have shown that a suboptimal density of class I or soluble anti-TCR mAb, which is not sufficient to fully activate CTL, can initiate TCR signaling which in turn enhances CD8 binding to non-Ag MHC class I and augments CTL adhesion and responses (6, 7, 9, 10). To test their effectiveness in initiating early TCR-dependent events in T cell activation, both the pDβ/DαLys to trigger CTL adhesion and degranulation response is not explained by an inability to bind to CD8 per se.

Figure 5. CD8 dependency of NP-specific CTL adhesion and degranulation in response to pDβ/Dαwt- and pDβ/DαLys-NP peptide complexes. Both pDβ/Dαwt (A and C) and pDβ/DαLys (B and D) were immobilized on plastic at a density of 0.1 μg/well, and pulsed with NP peptide at the concentrations shown. Clone 3/4 CTL cells were loaded into the wells at 2 × 10⁵ per well, and incubated at 37°C for 4 h. The CTL adhesion (A and B) and degranulation as measured by SE release (C and D) were carried out simultaneously. Anti-CD8 mAb inhibition of both adhesion and SE release were performed as described in Materials and Methods. Anti-CD8 mAb, 2.43, was used at 1:4 dilution of culture supernatant, and YTS169.4 was used at the concentration of 5 μg/ml. All results are the mean of triplicate wells.
Figure 6. Soluble anti-TCR-α/β mAb triggers CD8-dependent CTL binding and response to pD\textsuperscript{D}D\textsuperscript{D}wt and the α3 domain mutant, pD\textsuperscript{D}D\textsuperscript{D}Lys. The pD\textsuperscript{D}D\textsuperscript{D}wt and pD\textsuperscript{D}D\textsuperscript{D}Lys were separately immobilized on the wells at the indicated densities. The K\textsuperscript{b} alloreactive clone 11 CTL were resuspended with the anti-TCR-α/β mAb, H57.597, at a concentration of 0.5 µg/ml immediately before loading into wells. For anti-CD8 mAb blocking, clone 11 cells were incubated at room temperature for 30 min with 2.43 mAb (1:4 dilution of culture supernatant) before the addition of anti-TCR mAb. 1.5 \times 10^6 clone 11 cells were loaded into wells and incubated at 37°C for 4 h. Clone 11 binding (A) and SE release (B) of clone 11 cells were determined. Results were expressed as the mean ± SD. Clone 11 binding to BSA (3.9%) was treated as background and subtracted. Clone 11 without soluble anti-TCR-α/β stimulation bound to 0.15 µg/well input of both pD\textsuperscript{D}D\textsuperscript{D}wt and pD\textsuperscript{D}D\textsuperscript{D}Lys to a similar background level as clone 11 binding to BSA (data not shown). U, pD\textsuperscript{D}D\textsuperscript{D}wt; I, pD\textsuperscript{D}D\textsuperscript{D}Lys; O, pD\textsuperscript{D}D\textsuperscript{D}wt + 2.43; O, pD\textsuperscript{D}D\textsuperscript{D}Lys + 2.43.

Figure 7. The pD\textsuperscript{D}D\textsuperscript{D}wt and pD\textsuperscript{D}D\textsuperscript{D}Lys α3 domain mutant function equally well as CD8 accessory ligands for augmenting CTL binding and SE release when coimmobilized with suboptimal specific alloantigen. Purified K\textsuperscript{b} isolated from EL4 cells was immobilized on plastic at a suboptimal density of 0.02 µg/ml alone or together with the pD\textsuperscript{D}D\textsuperscript{D}wt or pD\textsuperscript{D}D\textsuperscript{D}Lys at the indicated densities. Clone 11 binding (A) and SE release (B) in response to coimmobilized suboptimal K\textsuperscript{b} and pD\textsuperscript{D}D\textsuperscript{D}wt (open squares) or pD\textsuperscript{D}D\textsuperscript{D}Lys (open circles) at various densities were determined. Binding of clone 11 to immobilized suboptimal K\textsuperscript{b} alone was 4.8%. Clone 11 SE release to suboptimal K\textsuperscript{b} Ag was at the background level (data not shown). Effects of the anti-CD8 mAb, 2.43, on suboptimal K\textsuperscript{b} Ag-triggered CTL binding (A) and enhanced degranulation response (B) to coimmobilized pD\textsuperscript{D}D\textsuperscript{D}wt (open squares) or pD\textsuperscript{D}D\textsuperscript{D}Lys (solid circles) were also examined. Results are expressed as mean of triplicate wells and variation is <10% of the mean. U, pD\textsuperscript{D}D\textsuperscript{D}wt; O, pD\textsuperscript{D}D\textsuperscript{D}Lys; I, pD\textsuperscript{D}D\textsuperscript{D}wt + 2.43; O, pD\textsuperscript{D}D\textsuperscript{D}Lys + 2.43.

Discussion

In the present report, we describe the direct comparison of wild-type MHC class I molecules with those bearing a Glu227→Lys α3 domain mutation previously shown to substantially diminish or abrogate CD8-dependent CTL lysis (4, 13, 14, 24), in mediating Ag-specific, CD8-dependent CTL clone adhesion and response. This was explored using purified MHC molecules on solid phase, where the density of class I and the number of peptide antigen-class I complexes were varied in a controlled manner. The number of peptide-class I complexes formed on solid phase with the immobilized wild-type and mutant class I by addition of exogenous peptide antigen are likely to be very similar, if not identical, since the addition of exogenous peptide antigen induces peptide-dependent conformational epitopes to an equivalent extent on wild-type and α3 domain mutated class I molecules expressed on endogenous peptide loading defective T2 cells (24). Furthermore, the acid-eluted endogenous peptides of K\textsuperscript{b} and D\textsuperscript{b} molecules...
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with their respective α3 mutant counterparts appear identical, suggesting that the point mutation in the α3 domain does not affect peptide binding (24, 34), despite the observation that the Asp227→Lys substitution in another class I, Ld, diminishes the interaction of this class I molecule with the endoplasmic reticulum peptide transporter, TAP, during biosynthesis (35).

We found the maximal CTL adhesion and degranulation response to be at least threefold greater to immobilized purified wild-type as opposed to mutant class I-presenting molecules pulsed with peptide. The mutant class I molecules did not achieve >10% of input CTL binding, which was only observed at high concentrations of antigenic peptide. These results are consistent with the differences in target cell lysis and degranulation responses we found with the same CTL clone in response to wild-type and mutant class I-bearing target cells. In experiments not easily approached using target cells, we show that in the presence of saturating peptide antigen, it requires an 8-16-fold higher density of immobilized mutant class I molecules to achieve the same level of CTL adhesion and response as with molecules bearing a wild-type α3 domain. Surprisingly, we observed CTL avidity-enhanced CD8 binding induced by soluble anti-TCR mAb or alloantigen not only to class I bearing a wild-type α3 domain but also to class I α3 mutants which lack a critical CD8 binding site. Furthermore, additional signaling associated with avidity-enhanced CD8 binding was only modestly reduced or undiminished with the α3 domain mutant class I relative to wild-type molecules in the two experimental systems used. In contrast, when incubated with peptide antigen, the mutant class I molecules were unable to trigger TCR-dependent signals necessary for the induction of avidity-enhanced CD8 binding to coimmobilized non–antigen class I.

Several amino acid substitutions in the acidic loop of the conserved α3 domain of class I disrupt CD8-dependent CTL antigen recognition (4, 13–15, 33). This is likely to be due to interference with CD8 coreceptor function since the mutagenesis of the antigen-presenting class I, the ligand for the TCR, blocks response despite the presence of non–Ag class I on the same cell membrane, which in theory could serve as accessory ligands for CD8. More direct evidence for CD8 serving a coreceptor adhesive role has been provided recently by Luescher et al. (10) who examined CTL interactions with soluble wild-type and α3 mutant Kd class I molecules. Using photoaffinity labeling techniques they directly demonstrated that CD8 can serve as a coreceptor by significantly strengthening cell surface TCR binding to soluble Kd on an allo-specific CTL clone and this interaction was shown to be inhibited by a Lys substitution for the Asp227 in Kd. However, there was no analysis of functional responses possible in this system to determine the relationship of binding to response, since class I in soluble monoclonal form is not a stimulus for T cell responses. Our studies with solid phase class I provide a quantitative comparison of class I molecules bearing wild-type or the α3 domain mutation for CTL adhesion and response. Our data are consistent with and strengthen the conclusions of previous reports suggesting that the Glu227 mutation disrupts CD8 coreceptor interactions, as our results reveal that CD8 coreceptor engagement with peptide antigen–class I complexes is necessary for the TCR-dependent expression of avidity-enhanced CD8 binding to non–Ag class I and CD8 accessory function by CTL.

Binding and response to class I-bearing cells or surfaces by CTL appears to be a dynamic multistep process controlled by the TCR (8). For example, no detectable CTL binding is observed to immobilized non–Ag class I in the absence of a TCR stimulus (6, 7), thus CD8 interaction with class I is likely to be minimal in the absence of TCR triggering. However, if the TCR is engaged with immobilized specific antigenic class I, high avidity CD8 accessory interaction with coimmobilized non–Ag class I is observed and this results in the delivery of additional signals leading to phosphatidylinositol hydrolysis and degranulation of CTL (6, 7, 36). That this process is likely to be sequential is supported by the observation that pretreatment of CTL with low concentrations of soluble antibodies to the TCR used...
to mimic antigen recognition, triggers subsequent high avidity CD8 binding to class I (6, 7). The CD8 avidity enhancement is blocked by inhibitors of tyrosine phosphorylation suggesting that this process is dependent on intracellular signaling (6, 36). Since the α3 domain mutant class I is defective in supporting appropriate triggering through the TCR, for CD8 binding in the presence of an excess of coimmobilized non-Ag class I (Fig. 8), our results suggest a sequential model of CD8 receptor functions. In this model, the Glu227 of H-2 class I is essential for early low avidity CD8 coreceptor function in stabilizing TCR engagement with class I and facilitating initial TCR activation signals, but for subsequent avidity-enhanced CD8 accessory molecule recognition and function which may involve additional binding sites on class I molecules. We do not exclude the possibility that when CD8 undergoes avidity enhancement it may also augment CD8 coreceptor function, but the expression of activated CD8 binding, regardless of whether the class I ligand is presenting the peptide antigen or not, would still be dependent on initial low avidity CD8 interactions.

It has been difficult to determine the relative contributions of CD8 coreceptor and accessory functions to CTL activation. However, since mutagenesis of the α3 domain of the specific Ag-presenting class I molecule (potential CD8 coreceptor ligand) can ablate CD8-dependent CTL reactivity even in the presence of endogenous non-Ag class I (potential CD8 accessory ligand), it has been suggested that CD8 functions much more efficiently as a coreceptor than as an accessory molecule (15, 24). Results in the present report indicate that this simple interpretation can be significantly revised and clarified. Our data support the conclusion that the induction and expression of avidity-enhanced CD8 accessory function is simply dependent on an initial CD8 coreceptor priming function and CD8 accessory interactions can be critical for CTL responsiveness at low antigen density. Thus both CD8 coreceptor and accessory functions are important for CTL activation. Without the initiating TCR-dependent CD8 coreceptor activity which is prevented or greatly diminished by the Glu227→Lys mutation, however, no avidity enhancement of CD8 occurs in response to α3 mutant class I–peptide antigen complexes and no subsequent CD8 accessory function can be observed. Therefore, our results provide an explanation for why little or no response is observed when peptide antigen is presented by the α3 domain mutant class I, despite the presence of non-Ag class I that in principle might be expected to compensate as CD8 accessory ligands for deficiencies in the α3 mutated class I presenting molecules.

Interestingly, we found that the α3 domain mutant class I molecule is still an effective ligand for avidity-enhanced CD8 binding, however it is unclear at present how CD8 binds the mutant molecules. This is hampered by the lack of understanding regarding how the avidity of CD8 is modulated by TCR triggering. It is clear from cell transfection and adhesion assays that the conserved α3 domain residues 222–229 acidic loop that includes Glu/Asp227 is a binding site on class I for CD8α homodimers when CD8α is overexpressed (15). Independent confirmation of an α3 binding site for CD8α was provided by Fayen et al. (37) who showed that the human CD8α IgV domain and the α3 domain of HLA-A2.1 can interact in soluble and immobilized forms and thus these domains of CD8 and class I, respectively, are sufficient for binding in vitro. It is possible that avidity-enhanced CD8 binding to mutant class I is still to the acidic loop of class I α3 domain, however its binding may be less dependent than CD8 in its low avidity state on the presence of Glu227. We have reported previously that avidity-enhanced CD8 binding is diminished by agents that inhibit cytoskeletal modifications (38). A consequence of cytoskeletal changes in response to TCR triggering may be an increase in cell spreading resulting in an increased surface area of contact. By increasing the area of contact, CTL would increase the quantity of CD8 that might engage class I on a target cell surface or on solid phase and the increased number of interactions of CD8 with class I between the two surfaces may be sufficient to support stable binding. A second consequence of cytoskeletal rearrangements induced by TCR triggering may be CD8 microclustering as found with the Cβ3i receptor (39), increasing the multivalency of CD8 and in turn increasing the avidity but not necessarily the affinity of CD8 for class I. In both of these scenarios, avidity increases between the CD8 and class I–bearing surfaces may compensate for reductions in CD8 affinity from the Glu227→Lys mutation without a change in CD8 binding site on class I.

It has recently been reported by Sun et al. (17) using transfection and overexpression systems that substitutions in the α2 domain of HLA class I can disrupt cell–cell adhesion mediated by human CD8α/α, suggesting that CD8 may have additional contact or binding sites on class I outside the α3 domain. Furthermore, recent studies using exon shuffling of domains between human and mouse class I genes, have provided evidence to suggest that the α2 domain is likely to influence CD8 interactions with class I in T cell development and mature CD8+ T cell reactivity (40, 41). Although additional CD8 binding sites on class I may be involved in basal or low avidity CD8 binding, CD8 interaction with these additional sites may be enhanced upon TCR triggering and support activated CD8 binding despite the Glu227→Lys mutation.

The preceding discussion has not considered structural changes of CD8 that may result from TCR triggering. It is conceivable that the CD8 heterodimer undergoes a conformational change resulting from TCR–triggered intracellular signals, which enhance CD8 affinity for the α3 acidic loop or alternatively allows CD8 to bind additional sites on class I. This may provide an explanation for our observation of activated CD8 adhesion to the mutant molecules. In this context, Meyerson et al. (42) have found that the presence of the membrane proximal connecting peptide or stalk of CD8 reduces NH2-terminal CD8α IgV domain binding to the α3 domain and the binding of purified CD8α in soluble form to class I is temperature sensitive.
These results could suggest that a specific or preferred conformation of CD8 or class I may be necessary for binding. Perhaps TCR-triggered changes in CTL lead to the induction or stabilization of appropriate CD8 conformation(s) for class I binding. In contrast to the report of Meyerson et al. and others involving CD8α/α homodimers, our study examines class I adhesion by CD8α/β heterodimers which are typically expressed on mature murine CD8+ T cells. Expression or lack thereof, of the CD8 β chain has been shown to substantially influence T cell thymic maturation (43-45) and CD8 β undergoes structural changes depending on T cell differentiation and activation state (46). It is less clear whether the CD8 β chain directly participates in mature T cell recognition of class I (47–49), however it remains to be determined whether the CD8 β chain may influence or possibly regulate basal or avidity-enhanced CD8 binding to class I.

We show that the purified Glu227→Lys α3 domain mutant molecules bound with peptide antigen are very poor ligands for CTL adhesion and response, yet there is still some low level CTL reactivity to these mutant class I-antigen complexes. It is not clear how the CD8–dependent CTL clone that has been shown to substantially influence T cell thymic maturation (43–45) and CD8 α undergoes structural changes depending on T cell differentiation and activation state (46). It is less clear whether the CD8 β chain directly participates in mature T cell recognition of class I (47–49), however it remains to be determined whether the CD8 β chain may influence or possibly regulate basal or avidity-enhanced CD8 binding to class I.

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References


Sun, J., D.J. Leahy, and P.B. Kavathas. 1995. Interaction between CD8 and major histocompatibility complex (MHC) class I mediated by multiple contact surfaces that include the α2 and α3 domains of MHC class I. J. Exp. Med. 182:1275–1280.


Detmers, P.A., S.D. Wright, E. Olsen, B. Kimball, and Z.A.


