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

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TCR (9, 11, 12). When the 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 either by strengthening the interactions between CTL receptors and their ligands, or transducing additional signals for T cell activation (6–10).

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 D^d α3 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 α3 domain of HLA class I molecules is a binding site for human CD8α/α 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/α 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 D^bα1α2 or K^bα1α2 and D^dα3 domains, in which the D^dα3 domain 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-triggered 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 $IgG2_a$ mAb recognizing H-2 D^b molecules, B22.249 (18), was a gift from Dr. U. Hammerling (Memorial Sloan-Kettering Cancer Center, New York). The K^b-specific mAb, Y3 ($IgG2_b$) (19), and D^d-specific mAbs, 34-5-8s ($IgG2_a$) (20) and 34-2-12s ($IgG2_a$) (20) were produced from their respective hybridomas obtained from American Type Culture Collection (ATCC; Rockville, MD). The rat anti-mouse CD8-producing hybridoma, 2.43 ($IgG2_b$) (21) was obtained from ATCC, and the YTS169.4 mAb (rat $IgG2_b$) (22) was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). The hamster anti-mouse TCRα/β mAb, H57-597 (23), was purchased from Pharmingen (San Diego, CA).

Transfectant Cell Lines Expressing Chimeric H-2 Class I with Wild-Type or Mutant α3 Domains. M12 ($H\text{-}2^d$), a B lymphoma cell line, was transfected with chimeric class I genes as described (13, 24, 25). Exon shuffled genes consisting of the α1 and α2 domain of the H-2 D^b or K^b genes and either the wild-type or Glu227→Lys substituted α3 domain of the H-2 D^d (13) were transfected into the M12 cell line by electroporation, and are referred to herein as M12.D^b/D^dwt, M12.D^b/D^dLys, M12.K^b/D^dwt, and M12.K^b/D^dLys, respectively.

Cloned CTLs. Clone 3/4 is specific for H-2 D^b and the influenza nucleoprotein (NP)[1] (365–380) 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 NH_2 terminus (Y-ASNENMETM) that was synthesized and purified at Multiple Peptide Systems (San Diego, CA). The K^b-allo-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, D^b/D^dwt, D^b/D^dLys, K^b/D^dwt, and K^b/D^dLys were purified from $0.5–1.0 \times 10^{10}$ transfectant cells by immunoaffinity chromatography as described (27, 28) with modifications. B22.249 (D^b α1; 29) and Y3 (K^b α2; 30) mAb columns were used for immunoaffinity purification of D^b/D^d and K^b/D^d 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 transfectant cells were passed over the B22.249 or Y3 columns. Columns were washed with 0.1% deoxy cholate (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 Na_2CO_3, 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 immunoaffinity-purified H-2 class I used in this study, including K^b and D^b isolated from EL4 cells and K^k isolated from RDM-4, as well as I-E^d 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 serine esterase (SE) activity released into the medium with the *N'*-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) assay as described (32). Stimulator cells were pulsed with the NP peptide for 30 min at RT, washed three times with 2% FCS-RPMI, and 3×10^5 stimulator cells were incubated with 10^5 cloned CTL for 4 h. 20 μl of supernatant was recovered and the BLT reactions proceeded for 20 min, whereupon the OD_{405nm} was determined. SE release is calculated as $\Delta OD_{405nm} = OD_{405nm}$ (CTL + stimulators) − OD_{405nm}(CTL alone).

CTL adhesion and degranulation responses to purified class I and coimmobilized proteins have been described previously in

[1] *Abbreviations used in this paper:* NP, influenza nucleoprotein; pD^b, D^b molecules purified from EL4 cells; pD^b/D^d Lys, purified chimeric D^b (α1α2) and D^d (α3 with Glu227→Lys substitution) molecules; pD^b/D^dwt, purified chimeric D^b (α1α2) and D^d (α3 wild type) molecules; pK^b/D^dLys, purified chimeric K^b (α1α2) and D^d (α3 with Glu227→Lys substitution) molecules; pK^b/D^dwt, purified chimeric K^b (α1α2) and D^d (α3 wild type) molecules; pK^b, K^b molecules purified from EL4 cells; SE, serine esterase.

detail (7, 27, 32). 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 ^{51}Cr-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, 10^4 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 dilution 1:4 of the culture supernatant and purified YTS169.4 was used at 5 μg/ml.

Results

Antigen-specific, CD8-dependent 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 α1α2 domain and D^d α3 domain. The D^d α3 domain is either the wild-type or mutant with a Glu227→Lys substitution (13, 24, 25). Both the transfectants, M12.D^b/D^dwt or M12.D^b/D^dLys, express comparable levels of the hybrid D^b molecules as detected by FACS® analysis using a D^b-specific mAb, B22.249 (data not shown). The target cells were pulsed with NP peptide at different concentrations, and cytolysis was measured by ^{51}Cr release. As expected, and similar to our previous report (24), M12.D^b/D^dwt target cells were killed by clone 3/4 to a level comparable to EL4 (H-2^b), which expresses natural D^b molecules (Fig. 1 *A*). Clone 3/4 also lysed M12.D^b/D^dLys, with two significant differences compared

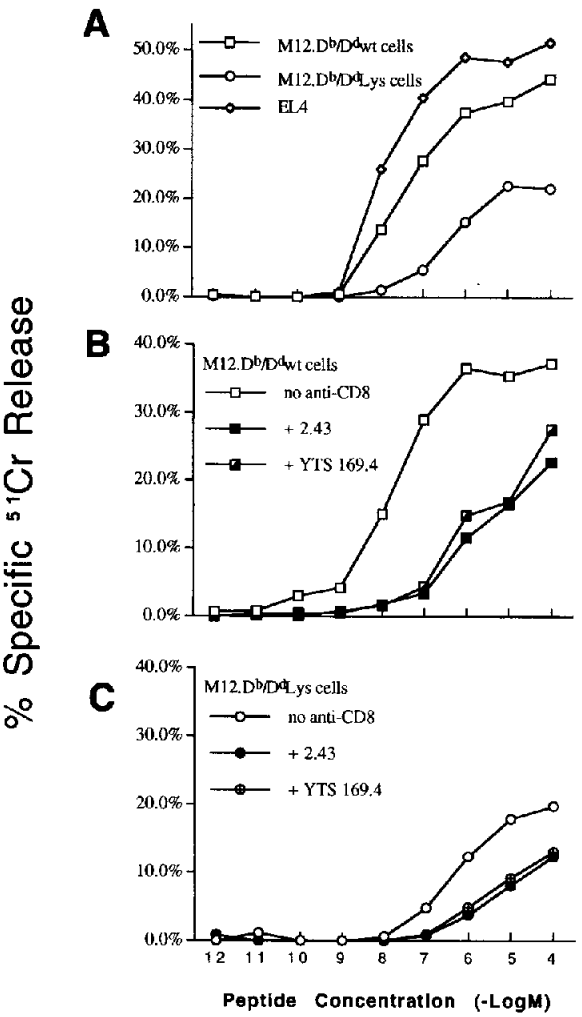

Figure 1. Antigen-specific, $CD8^+$ CTL clone lysis of target cells bearing MHC class I molecules with wild-type or mutant α3 domains. (*A*) Lysis of M12.D^b/D^dwt, M12.D^b/D^dLys transfectant cells and EL4 cells by an influenza NP-specific CTL, clone 3/4. ^{51}Cr-labeled targets were pulsed with the influenza NP peptide (Y-NP366-374) at the indicated concentrations. 10^4 targets were incubated together with clone 3/4 effector cells at a 5:1 E/T ratio at 37°C for 5 h. Effects of CD8-specific mAbs on clone 3/4 CTL killing of M12.D^b/D^dwt (*B*) and M12.D^b/D^dLys (*C*) cells were determined. Clone 3/4 effector cells were incubated with anti-CD8 mAbs at RT for 30 min before assay with ^{51}Cr-labeled, NP peptide–pulsed targets. 2.43 mAb was used as 1:4 dilution of culture supernatant and purified YTS169.4 used at the concentration of 5 μg/ml. No lysis of targets was seen without NP peptide pulsing (data not shown). Percent specific lysis is expressed as the mean of triplicate wells and variation is <8% of the mean.

to M12.D^b/D^dwt lysis (Fig. 1 *A*). First, the maximal levels of lysis of M12.D^b/D^dLys (20%) were only one-third to one-half of that of M12.D^b/D^dwt (40–50%). Second, the concentration of peptide required to obtain significant lysis was >100-fold higher in M12.D^b/D^dLys than in M12.D^b/D^dwt. 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^dLys gene was substantially lower and required higher concentrations of OVA

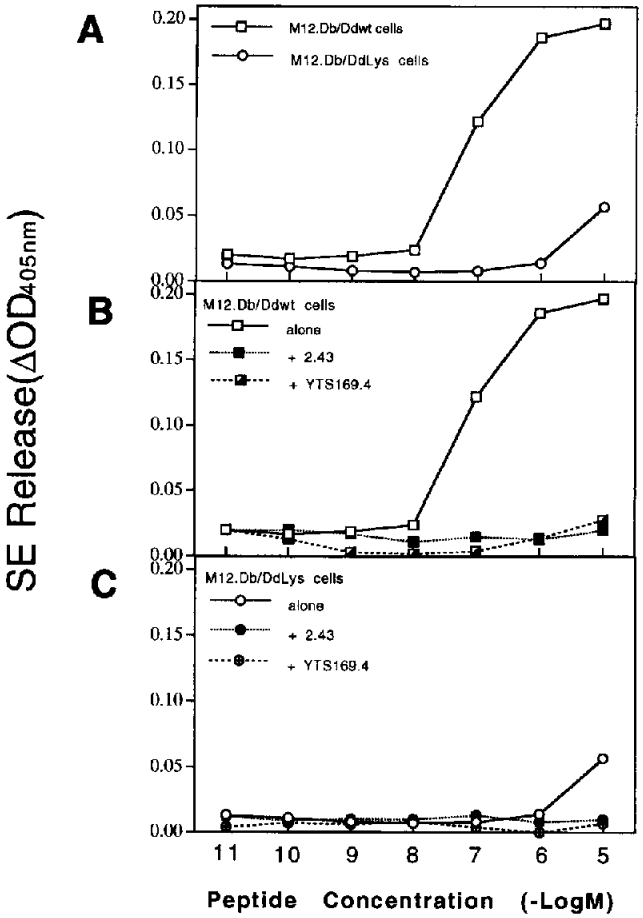

Figure 2. Antigen-specific CTL clone degranulation responses to cells bearing wild-type or α3 domain–mutated class I molecules. (*A*) Degranulation of an influenza NP-specific CTL clone, 3/4, in response to M12.D^b/D^{d}wt and M12.D^b/D^{d}Lys transfectants. Both transfectants were pulsed with NP peptide at the indicated concentrations. After washing, 3×10^5 transfectant cells were incubated with 10^5 clone 3/4 cells at 37°C for 4 h. Effects of anti-CD8 antibodies on clone 3/4 degranulation in response to M12.D^b/D^{d}wt (*B*) and M12.D^b/D^{d}Lys (*C*) cells were also determined. Clone 3/4 cells were incubated with anti-CD8 antibodies at room temperature for 30 min before incubating with the peptide-pulsed transfectant cells. Anti-CD8 mAb, 2.43, was used at 1:4 dilution of culture supernatant, and YTS169.4 used at the concentration of 5 μg/ml. In all the panels, SE release is expressed as the mean of triplicate wells and variation is <10% of the mean.

peptide than target cells transfected with the K^b/D^{d}wt gene (24). M12.D^b/D^{d}wt cell lysis by clone 3/4 CTL was almost completely blocked by CD8α-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^{d}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^{d}wt cells triggered a substantially stronger SE release than M12.D^b/D^{d}Lys cells, and these responses were peptide dose dependent (Fig. 2 *A*). Clone 3/4 degranulation responses triggered by both M12.D^b/D^{d}wt and M12.D^b/D^{d}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^b/D^{d}wt and pD^b/D^{d}Lys*

mAbs*	Specificity	pD^b/D^{d}wt	pD^b/D^{d}Lys
B22.249	D^b α1	1.062 ± 0.052‡	1.092 ± 0.048
34-5-8s	D^d α1α2	0.003 ± 0.001	0.013 ± 0.002
34-2-12s	D^d α3	0.347 ± 0.032	0.009 ± 0.002
Y3	K^b α2	0.005 ± 0.001	0.017 ± 0.001

ELISA reactivity of pD^b/D^{d}wt and pD^b/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-RPMI at 1:4 dilution.
‡Results were expressed as mean OD_{490nm} ± SD of triplicate wells.

Direct Comparison of CTL Adhesion and Response to Purified D^b/D^{d}wt and D^b/D^{d}Lys Pulsed with NP Peptide Ag. FACS® analysis showed that both M12.D^b/D^{d}wt and M12.D^b/D^{d}Lys transfectant cells expressed other class I molecules, e.g., D^d (data not shown), and may express other cell surface accessory molecule ligands. To directly compare D^b/D^{d}wt- and D^b/D^{d}Lys-peptide complexes in CTL recognition and activation, we purified D^b/D^{d}wt and D^b/D^{d}Lys molecules for use in solid-phase CTL recognition assays (7, 27, 32). Since B22.249 mAb recognizes an α1 domain epitope of D^b, independent of the α3 domain (29), it was used for immunoaffinity purifications of D^b/D^{d}wt and D^b/D^{d}Lys chimeric molecules. The purified D^b/D^{d}wt (pD^b/D^{d}wt) and D^b/D^{d}Lys (pD^b/D^{d}Lys) were analyzed using solid-phase ELISA (28) (Table 1). Both pD^b/D^{d}wt and pD^b/D^{d}Lys were strongly positive for the B22.249 (D^b α1) epitope; while negative for the 34-5-8s epitope (D^d α1α2). As expected, the pD^b/D^{d}wt, but not the pD^b/D^{d}Lys, was reactive with 34-2-12s (D^d α3) since the D^d Glu227→Lys substitution abrogates recognition by 34-2-12s (13, 14). All preparations were standardized by protein and ELISA assays.

The pD^b/D^{d}wt, pD^b/D^{d}Lys, and pD^b (isolated from EL4 cells) were immobilized separately on microtiter plate wells, pulsed with NP peptide at the concentrations indicated, and tested for their abilities to trigger H-2 D^b-restricted, NP-specific CTL (Fig. 3). As we have shown previously (7, 32), the NP-specific CTL clone 3/4 bound, and degranulated in response to, the immobilized pD^b-NP peptide complexes (Fig. 3). Clone 3/4 cells also bound to both pD^b/D^{d}wt and pD^b/D^{d}Lys, but the levels of binding and response are dramatically different (Fig. 3 *A*). We consistently observed that the maximal percentage of clone 3/4

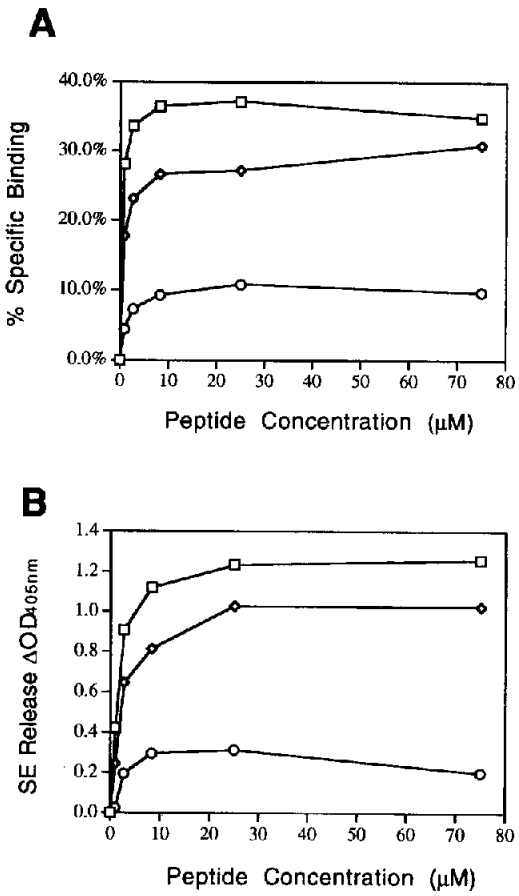

Figure 3. Comparison of influenza NP–specific CTL clone adhesion and SE release to immobilized pD^b/D^dwt and pD^b/D^dLys complexed with NP peptide. The pD^b/D^dwt, pD^b/D^dLys, and pD^b were immobilized at a density of 0.1 μg/well, and pulsed with NP peptide at the concentrations indicated. Clone 3/4 CTL were loaded at 2×10^5/well, and incubated at 37°C for 4 h. The CTL adhesion (*A*) and degranulation as measured by SE release (*B*) were determined simultaneously. Nonspecific binding (3.4%) was subtracted. Results are expressed as the mean of triplicate wells and variation is <10% of the mean. □, pD^b/D^dwt; ○, pD^b/D^dLys; ◇, pD^b.

CTL cells adhered to immobilized pD^b/D^dwt was more than threefold greater than to pD^b/D^dLys (38% compared with 10%). CTL binding to both pD^b/D^dwt and pD^b/D^dLys 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 pD^b/D^dwt than pD^b/D^dLys.

The pD^b/D^dwt and pD^b/D^dLys density dependence for CTL binding and degranulation was also investigated (Fig. 4). Both pD^b/D^dwt and pD^b/D^dLys 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*) was density dependent for pD^b/D^dwt and pD^b/D^dLys, with pD^b/D^dwt supporting a threefold greater maximal CTL binding (Fig. 4 *A*) and triggering a fivefold stronger maximal degranulation response (Fig. 4 *B*) than pD^b/D^dLys. When a saturating amount of peptide was used to pulse both the pD^b/D^dwt and pD^b/D^dLys at or below saturating densities (0.025 μg/well or less), 8–16-fold more pD^b/D^dLys molecules were required to reach the same level of CTL binding to pD^b/D^dwt pulsed with peptide (Fig. 4 *A*). About a 10-fold higher density of pD^b/D^dLys was needed to induce a similar level of clone 3/4 SE release as needed for pD^b/D^dwt, with saturating peptide (Fig. 4 *B*). An ELISA of immobilized pD^b/D^dwt and pD^b/D^dLys done in parallel using D^b-specific mAb, B22.249, confirmed that at the same input of purified class I protein, the immunoreactive D^b epitopes for both pD^b/D^dwt and pD^b/D^dLys 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 pD^b/D^dwt and pD^b/D^dLys were significantly inhibited by anti-CD8 mAb, 2.43 and YTS169.4, suggesting that not only the pD^b/D^dwt, but also the low level pD^b/D^dLys-triggered responses are CD8 dependent, thus the pD^b/D^dLys 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 Glu227→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 pD^b/D^dwt and pD^b/D^dLys 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 and cosignaling function, resulting in CTL degranulation (6–8). As shown above, the D^b/D^dLys mutant class I could not effectively activate CTL. One possibility that could contribute to this result would be that the D^b/D^dLys molecule does not serve as an effective ligand for TCR-activated CD8 as compared to D^b/D^dwt molecules. To test this possibility, we stimulated the K^b-specific CTL clone 11 with soluble anti-TCR mAb (H57.597), and determined whether the CTL bind to immobilized pD^b/D^dwt or pD^b/D^dLys, non–Ag class I molecules in this system (6–8). Clone 11 triggered by fluid-phase anti-TCR-α/β bound substantially to both pD^b/D^dwt and pD^b/D^dLys in a density-dependent manner (Fig. 6 *A*), but not to the purified

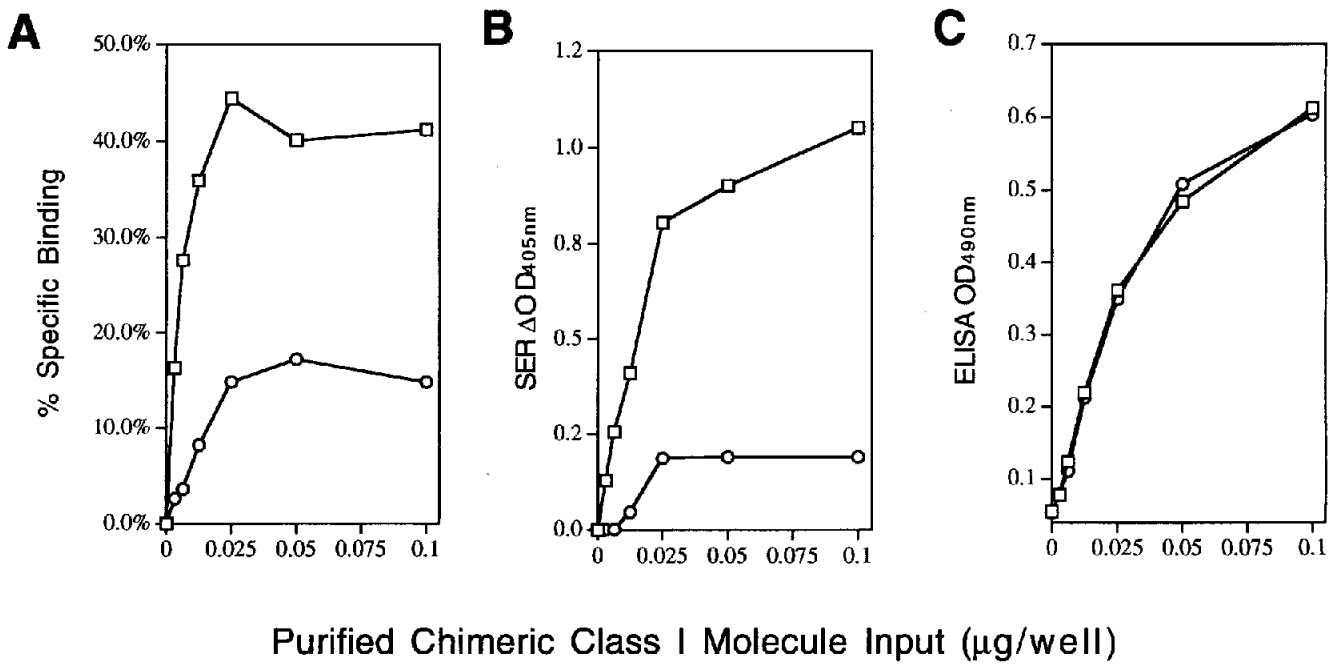

Figure 4. Density dependence of isolated wild-type and mutated α3 domain class I for Ag-specific CTL adhesion and SE release response. The pD^b/D^dwt (*squares*) and pD^b/D^dLys (*circles*) were immobilized on microtiter plate wells at the indicated densities, and pulsed with NP peptide at a saturating concentration of 75 μM. NP-specific clone 3/4 CTL cells were loaded into the wells at 2×10^5/well, and incubated at 37°C for 4 h. The CTL adhesion (*A*) and degranulation as measured by SE release (*B*) were done simultaneously from the same wells. Nonspecific binding (4.1%) was subtracted. (*C*) Solid-phase ELISA of pD^b/D^dwt and pD^b/D^dLys molecules. The pD^b/D^dwt (*squares*) and pD^b/D^dLys (*circles*) molecules used in binding (*A*) and SE release (*B*) assays were titrated on the plates at the same densities. Their ELISA reactivities were determined by using a D^b-specific mAb, B22.249, and results are expressed as the mean of duplicate wells.

murine class II molecule, $I\text{-}E^d$ (data not shown). We also compared the ability of the pD^b/D^dwt and pD^b/D^dLys molecules to provide CD8-dependent cosignals for CTL degranulation in conjunction with soluble anti-TCR antibody. Both pD^b/D^dwt and pD^b/D^dLys 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^b/D^dLys is comparable to or slightly lower (70–90%) than that of pD^b/D^dwt 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^b/D^dwt or pD^b/D^dLys 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^b/D^dwt and K^b/D^dLys 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^b/D^dwt and D^b/D^dLys (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^b/D^dwt or pD^b/D^dLys can serve as non–Ag CD8 accessory ligands to enhance CTL binding and degranulation. Both pD^b/D^dwt and pD^b/D^dLys were titrated and coimmobilized with a suboptimal density of purified K^b alloantigen on the wells. The binding and SE release of the K^b allo-specific clone 11 in response to suboptimal K^b and coimmobilized pD^b/D^dwt or pD^b/D^dLys were determined (Fig. 7). Augmentation of clone 11 binding (Fig. 7 *A*) and triggering of degranulation (Fig. 7 *B*) facilitated by the coimmobilized pD^b/D^dwt or pD^b/D^dLys was to very similar levels throughout the class I density curves. As expected, the enhanced binding and augmented SE release facilitated by both pD^b/D^dwt and pD^b/D^dLys 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^b/D^dwt or pK^b/D^dLys molecules are equally effective as avidity-enhanced CD8 ligands to enhance CTL binding and degranulation when the D^b-restricted, NP-specific CTL clone 3/4 is triggered by suboptimal D^b–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

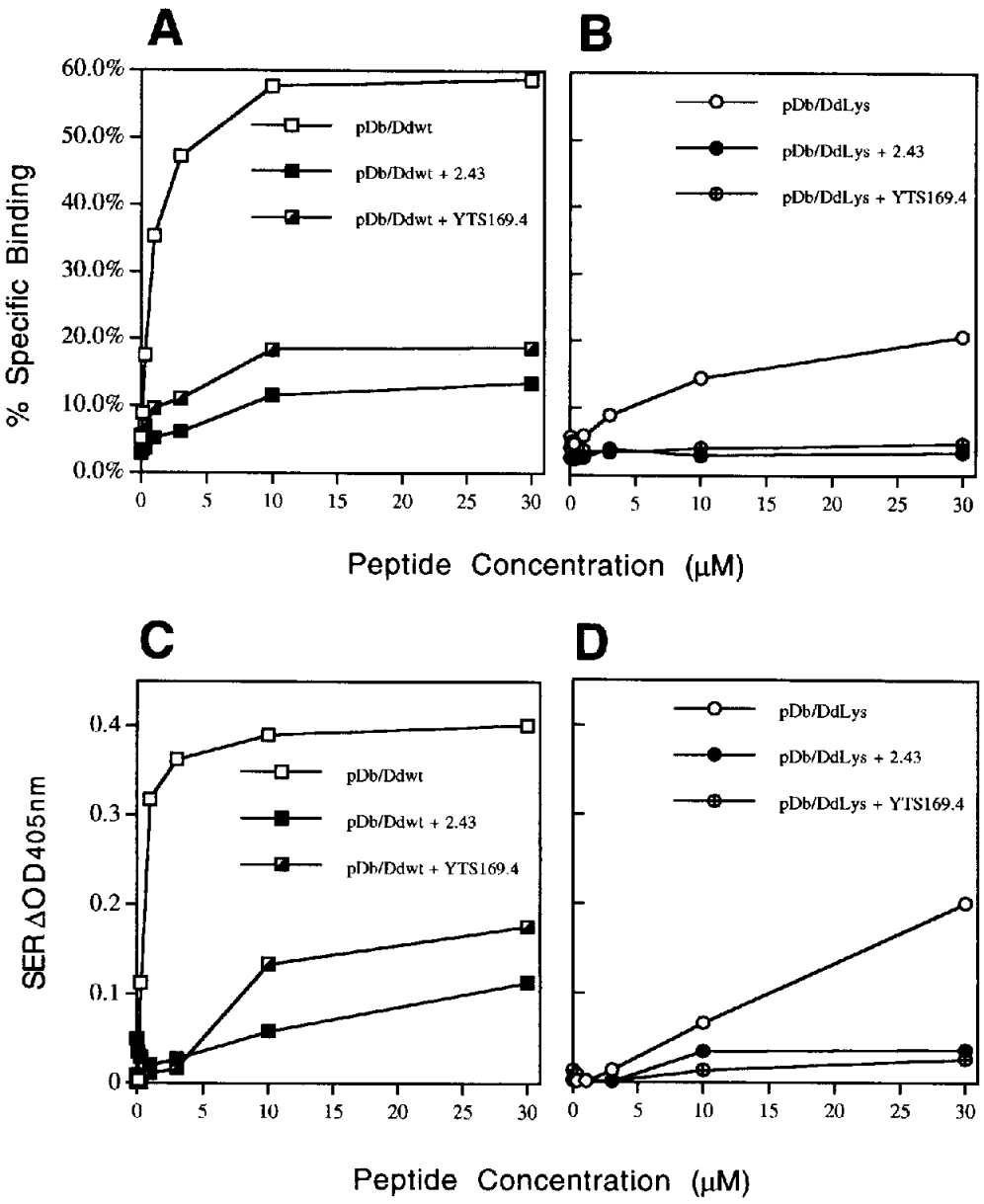

Figure 5. CD8 dependency of NP-specific CTL adhesion and degranulation in response to pD^b/D^dwt- and pD^b/D^dLys-NP peptide complexes. Both pD^b/D^dwt (*A* and *C*) and pD^b/D^dLys (*B* and *D*) were immobilized on plastic at a density of 0.1 μg/well, and pulsed with NP peptide at the concentrations shown. Clone 3/4 CTL cells were loaded into the wells at 2×10^5 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.

pD^b/D^dLys to trigger CTL adhesion and degranulation response is not explained by an inability to bind to CD8 per se.

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 D^b/D^dLys molecules can serve as effective non–Ag class I ligands for TCR-activated, avidity-enhanced CD8 (Figs. 6 and 7), we investigated whether D^b/D^dLys–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 pD^b/D^dwt and pD^b/D^dLys were titrated and coated on plastic with or without coimmobilization of a non–Ag class I molecule, K^k. After pulsing the immobilized class I with NP peptide, clone 3/4 CTL were added to the wells and their enhanced binding and SE release to coimmobilized non–Ag class I were determined (Fig. 8). Coimmobilized K^k greatly augmented clone 3/4 adhesion (Fig. 8 *A*) and SE release (Fig. 8 *B*) in response to pD^b/D^dwt-NP complexes. This effect was observed at the pD^b/D^dwt input as low as 0.0008 μg/well for enhanced binding (Fig. 8 *A*), and 0.006 μg/well for augmented degranulation (Fig. 8 *B*), while at these concentrations, pD^b/D^dwt alone does not trigger CTL adhesion or degranulation response. In contrast, over a range up to 0.05 μg/well input (approaching immobilization saturation), pD^b/D^dLys did not trigger CTL for enhanced bind-

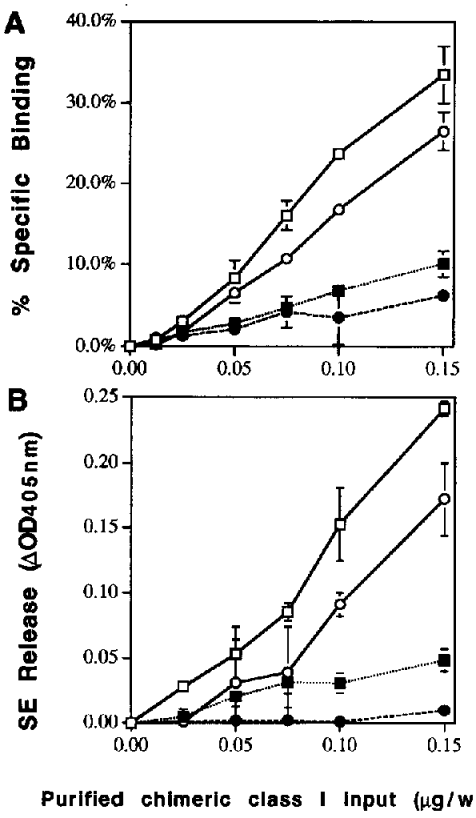

Figure 6. Soluble anti-TCR-α/β mAb triggers CD8-dependent CTL binding and response to pD^b/D^{d}wt and the α3 domain mutant, pD^b/D^{d}Lys. The pD^b/D^{d}wt and pD^b/D^{d}Lys were separately immobilized on the wells at the indicated densities. The K^b alloreactive clone 11 CTL were resuspended with the 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 × 10^5 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^b/D^{d}wt and pD^b/D^{d}Lys to a similar background level as clone 11 binding to BSA (data not shown). □, pD^b/D^{d}wt; ■, pD^b/D^{d}wt + 2.43; ○, pD^b/D^{d}Lys; ●, pD^b/D^{d}Lys + 2.43.

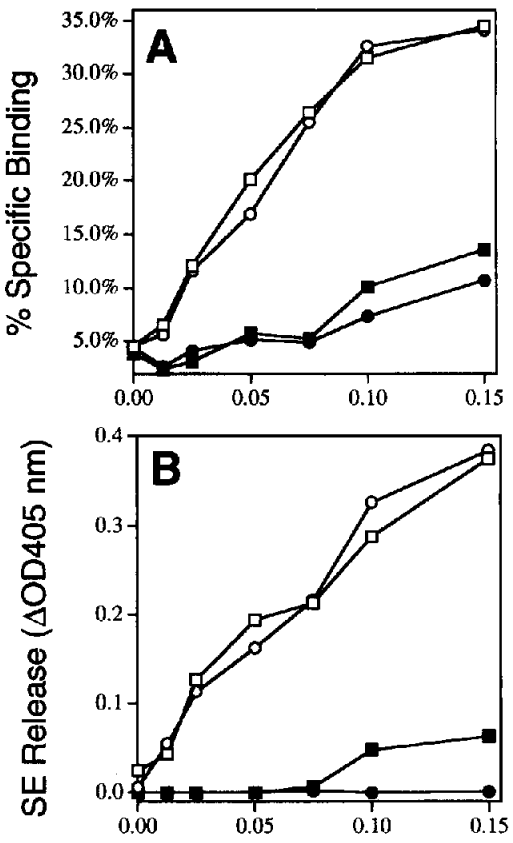

Figure 7. The pD^b/D^{d}wt and pD^b/D^{d}Lys α3 domain mutant function equally well as CD8 accessory ligands for augmenting CTL binding and SE release when coimmobilized with suboptimal specific alloantigen. Purified K^b isolated from EL4 cells was immobilized on plastic at a suboptimal density of 0.02 μg/ml alone or together with the pD^b/D^{d}wt or pD^b/D^{d}Lys at the indicated densities. Clone 11 binding (*A*) and SE release (*B*) in response to coimmobilized suboptimal K^b and pD^b/D^{d}wt (*open squares*) or pD^b/D^{d}Lys (*open circles*) at various densities were determined. Binding of clone 11 to immobilized suboptimal K^b alone was 4.9%. Clone 11 SE release to suboptimal K^b Ag was at the background level (data not shown). Effects of the anti-CD8 mAb, 2.43, on suboptimal K^b Ag-triggered CTL binding (*A*) and enhanced degranulation response (*B*) to coimmobilized pD^b/D^{d}wt (*solid squares*) or pD^b/D^{d}Lys (*solid circles*) were also examined. Results are expressed as mean of triplicate wells and variation is <10% of the mean. □, pD^b/D^{d}wt; ○, pD^b/D^{d}Lys; ■, pD^b/D^{d}wt + 2.43; ●, pD^b/D^{d}Lys + 2.43.

ing or SE release when coimmobilized with K^k (Fig. 8, *A* and *B*). These results indicate that the α3 domain mutation in MHC class I molecules likely affects initial low avidity or basal CD8 binding to class I–peptide Ag complexes which interferes with its CD8 coreceptor function, but not subsequent avidity-enhanced CD8 binding and accessory function.

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^b and D^b molecules

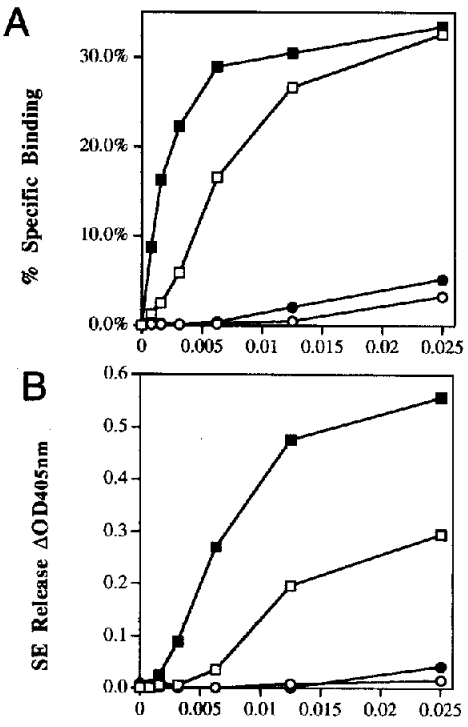

Figure 8. The pD^b/D^dLys–NP peptide complexes are defective in initiating TCR-triggered avidity-enhanced CD8 adhesion to non–Ag class I and CTL response. Both the pD^b/D^dwt (*squares*) and pD^b/D^dLys (*circles*) were titrated and immobilized separately on plastic with (*solid symbols*) or without (*open symbols*) the coimmobilization of a non–Ag class I molecule, K^k. Immunoaffinity purified K^k was used at a density of 0.20 μg/well. After pulsing with 30 μM NP peptide, 1.8×10^5 clone 3/4 CTL cells were loaded and incubated at 37°C for 4 h. Clone 3/4 binding (*A*) and SE release (*B*) were measured simultaneously. Nonspecific binding to BSA was 5.2% and subtracted. Clone 3/4 binding to immobilized K^k alone was 2.3%. □, pD^b/D^dwt alone; ■, pD^b/D^dwt + K^k; ○, pD^b/D^dLys, alone; ●, pD^b/D^dLys + K^k.

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, L^d, 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 K^d 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 K^d on an allo-specific CTL clone and this interaction was shown to be inhibited by a Lys substitution for the Asp227 in K^d. 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 monomeric 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 not 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 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 C3bi 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 NH_2-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 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 is responding to the mutant class I molecules, but responses are only found at relatively high concentrations of peptide and this is also true for peptide-pulsed target cells (Fig. 1 and 2; 24). The CTL responses to the mutant class I complexes on cells and solid phase however were partially or completely inhibited by CD8-specific antibodies, suggesting that CD8 is still participating in mutant class I molecule recognition. The combination of multiple low affinity TCR and residual low avidity CD8 interactions may be sufficient to support the low level binding and response observed with the mutant class I presenting molecules and peptide. Alternatively or additionally, at high mutant class I–antigen complex density there may be some minimal TCR-dependent signaling resulting in a small degree of avidity enhancement of CD8 binding, but if this occurs it is inefficient, since we only observe a low level of CTL binding and response.

The induction of avidity-enhanced CD8 binding requires tyrosine phosphorylation but not downstream signaling events such as phosphatidylinositol hydrolysis (6, 36). The specific intracellular signaling molecules associated with this phenomenon however have not been characterized. In contrast to APCs expressing wild-type class I antigen, cells expressing α3 domain mutant class I antigen fail to stimulate CTL phosphatidylinositol hydrolysis (25). Other intracellular changes that may be elicited in CTL upon engagement with the α3 domain mutant class I–peptide complexes have not been described. However, since the mutant molecule–peptide complexes fail to stimulate CTL avidity-enhanced CD8 binding, they may provide, by comparison with wild-type class I–peptide complexes, a means to identify CTL transmembrane signaling events resulting in avidity-enhanced CD8 binding.

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