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

By Jiaren Sun,* Daniel J. Leahy,‡ and Paula B. Kavathas*§

*From the *Department of Laboratory Medicine and Section of Immunobiology, Yale University School of Medicine, New Haven, Connecticut 06520-8035; ‡Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2185; § Department of Genetics, Yale University School of Medicine, New Haven, Connecticut 06520-8035*

Summary

The cell surface glycoprotein CD8 functions as a coreceptor with the TCR on cytotoxic T lymphocytes. Mutational analysis of the binding site of CD8 for MHC class I predicted that distinct surfaces of CD8 would interact with both the α2 and α3 domains of class I. Using a cell–cell adhesion assay, we identified three residues Q115, D122, and E128 in the α2 domain of class I critical for interaction with CD8. The side chains of these residues point towards a cavity formed by the α1/α2 platform, the α3 domain and β2-microglobulin (β2m) of class I. These residues were predicted to contact CD8 based on a bivalent model of interaction between one CD8α/α homodimer and two MHC class I molecules. These results therefore provide support for the model.

MHC class I molecules are highly polymorphic proteins that bind antigenic peptides and present them to T cells. The functional interaction of cytotoxic T cells with antigen-presenting cells requires the engagement of T cell receptor, in concert with CD8, with the MHC/peptide complex (1, 2). Following a large body of evidence suggesting a central role of CD8 in T cell signaling, activation and thymic selection, much interest has centered on the characterization of interaction between various components involved in T cell recognition (3, 4). It has become clear that whereas the TCR binds to the α1 and α2 domains of a class I–peptide complex, CD8 binds directly to the α3 domain (5–9).

A soluble form of CD8α/α homodimer was crystallized and its two Ig-like domains were found to share many structural similarities with their Ig counterparts (10). Mutational analysis of CD8α/α revealed that unlike Ig molecules in which the surface containing the CDR loops is exclusively used to recognize antigen, distinct surfaces of CD8α/α, one containing the CDR-like loops and the other comprised of the A and B β-strands, interact with class I (11). The discovery of a non-CDR surface as an important contact area on CD8α led us to postulate that CD8 interacted not only with the α3 domain but also with the α2 domain of class I. To test this hypothesis, we performed site-specific mutagenesis of class I and analyzed the mutants using a cell–cell adhesion assay (12). We identified three critical residues in the α2 domain, two located underneath the peptide-binding floor and one on a nearby loop, all with the side chains pointing towards a cavity (13). Thus, CD8α/α appears to interact both with the α2 and α3 domain of MHC class I.

Materials and Methods

Construction of Mutant HLA-A2 cDNAs. Site-specific mutagenesis was performed as described (14) using a cDNA template encoding HLA-A2010 (15). All mutations were confirmed by sequencing a 430 bp PflMI/NdeI fragment of the HLA-A2 gene encoding the α2 domain (NdeI/SmaI fragment encoding α3 in Q226A). Fragments were inserted into corresponding sites of wild type HLA-A2 in pBluescript II. HLA-A2 mutants were then subcloned into NotI/SalI sites in the plasmid vector EBO-pLPP containing an hygromycin B resistance gene (16).

Cells and Antibodies. C1R is a B cell line that lacks endogenous HLA-A, B genes thus expresses virtually no class I products. Wild-type and mutant A2 cDNA constructs were electroporated into C1R cells 960 μF 250V (17). All A2 transfectants (C1R-A2) were maintained in hygromycin B at 600–1,000 μg/ml, depending on levels of HLA-A2 expression. CHO cells either transfected (MT8.02) or mock transfected (MT8.C13) with the CD8 α-chain gene were provided by R. Salter (University of Pittsburgh) (12). MT8.02 and MT8.C13 CHO cell lines were grown in Ham's F12 medium lacking hypoxanthine (JRH Biosciences, Lenexa, KS) (6). High CD8 expression levels of MT8.02 were maintained

under 0.02 μM methotrexate amplification. mAb MA 2.1 (18) specific for HLA-A2 was used to stain all A2 transfectants. mAb TP25.99 specific for the α3 domain of class I was provided by S. Ferrone (New York Medical College) (16). Fluoresceinated goat anti–mouse IgG was used as a secondary antibody. The mAbs used for staining CD8α were FITC-conjugated OKT8 and Leu2a.

Adhesion Assay Determining Binding of HLA-A2 Mutants to CD8. The assay was modified from the method previously described (12). Briefly, 0.3×10^5 $CD8^+$ or $CD8^-$ CHO cells per well were plated. 1×10^6 C1R cells transfected with HLA-A2 were labeled with a fluorogenic esterase substrate Calcein AM (Molecular Probes, Eugene, OR). The cells were incubated in 1 ml PBS containing 10 μg Calcein AM at 37°C for 30 min. 3×10^5 labeled C1R cells were overlaid onto monolayers of CHO cells in triplicate. Bound C1R cells were lysed in 25 mM Tris/0.1% SDS after wash. Readings were taken with a fluorescence spectrophotometer at 495 nm (excitation) and 525 nm (emission) the next day. Measurement of fluorescence intensity of the labeled C1R cells before addition to the plated CHO cells indicated uniform labeling of C1R cells (data not shown).

Binding Assay of Mutant CD8α to Class I. The cell–cell binding assay was performed as described (11). Briefly, COS-7 cells firmly attached to a tissue culture dish were transfected using lipofectamine (Life Technologies, Inc., Grand Island, NY) with wild-type or mutant forms of CD8α. CD8α transfectants were tested for their ability to bind to the MHC class I^+ cell line UC (19). Since these UC cells were modified to constitutively express the firefly luciferase gene, the number of bound cells was determined by measuring the amount of luciferase activity in the cell extract. Expression levels of CD8 in COS-7 cells were checked in every experiment by flow cytometry of cells stained with FITC-conjugated mAb OKT-8 and Leu2a. Since the levels of cell surface expression varied slightly among wild-type and mutant forms of CD8, the binding of UC cells was normalized according to the following formula: 100 × % binding = ([mutant binding − vector binding]/mutant OKT8)/([wild-type binding − vector binding]/wild-type OKT8) (20).

Results and Discussion

To examine the effects of class I mutations on binding to CD8, we substituted an alanine residue in several areas within the α2 domain of class I. The majority of residues chosen for mutagenesis was based on our model of CD8 and class I interaction (11). All positions were surface accessible and many of them well conserved, reasoning that these residues are most likely to make contact with the class I molecule without rendering global structural alterations. Amino acids Q115 and D122 are on the peptide-binding floor with their side chains pointing away from the cleft (Fig. 1 *a*). Residues K127, E128, and D129 are located on the loop connecting β-strands 3 and 4 (S3 and S4) whereas S123, T134, and D137 are all on nearby S4. Another set of mutations, D102, L110, and R111 all located near the junction of S1 and S2, were based upon another molecular docking model (21). Residues E89 and E173 were chosen as potential controls for their likely inability to bind to CD8 based on our model. Wild-type and mutant forms of an HLA-A2 cDNA were subcloned into an EBO expression vector and transfected into C1R cells that lack endogenous HLA-A, B genes (17). All C1R-A2 mutants except for D129 expressed high levels of HLA-A2 with mean fluorescence intensities ranging from 90–110% of the wild-type transfectant (Fig. 1 *b*).

The A2 transfectants were tested for binding to CHO cells expressing the human CD8α gene, using a cell–cell adhesion assay. As shown in Fig. 2 *a*, fluorescence-labeled wild-type transfectants bound to the $CD8^+$ CHO cell monolayer to near saturation. The binding of HLA-A2 C1R transfectants to the $CD8^+$ CHO cells was mediated specifically by MHC class I and CD8 molecules since this interaction can be exclusively and completely inhibited by either anti-class I or anti-CD8 mAbs (5, 12). We included two mutants, A245V and Q226A of HLA-A2 as negative controls since these mutations in the α3 domain were previously shown to be responsible for negative CD8 binding phenotypes (5, 6). Neither of these two mutant transfectants bound to the $CD8^+$ CHO cells (Fig. 2, *a* and *b*).

Mutations at Q115, D122, and E128 showed no specific binding to CD8 (Fig. 2, *a* and *b*). This effect can not be attributed to levels of HLA-A2 expression by the various transfectants, since expression levels of the non-binders were equal to or greater than at least one of the CD8 binders (Fig. 1 *b* and data not shown). To exclude possible indirect effects on the α3 domain by conformational changes, we stained all mutants with mAb TP25.99 that binds specifically to the α3 domain of class I where the critical CD8 binding loop was located (22). Staining of all mutants with TP25.99 was similar to the wild-type transfectant (data not shown). These results suggest direct involvement of these three residues in contacting CD8 α-chain. While E128 appeared critical in binding, mutations at nearby K127 or distant E89 and E173 did not affect the interaction. Mutations S132A, T134A, and D137A on S4 did not affect binding (Fig. 2, *a* and *b*), nor did the exposed junction near S1 and S2 (D102A, L110A, R111A, and D102A/R111A), indicating that much of other exposed areas on the α2 domain of class I is not likely to be critical for interacting with CD8. Since our analysis did not score mutations that simultaneously resulted in a loss of surface expression and a reduced affinity for CD8 (i.e., D129, data not shown), the data resulted in description of only a group of critical residues. In cocrystals of human growth hormone and its receptor, a few contact amino acids clustered in a central region accounted for 85% of the binding free energy resulting from the alanine substitutions whereas all peripheral residues only contributed to 15% of the binding free energy (23).

To further define the A/B surface of CD8 that may contact the α2 domain of MHC class I, we mutated several solvent accessible residues on the A, B, and G β-strands of human CD8 to alanine. Once CD8 mutants were generated, a transient cell–cell adhesion assay was employed as described (11). In this assay, COS 7 cells expressing CD8 mutants were tested for their ability to bind to the class I^+ cell line UC that carries a firefly luciferase gene. Replacement of R4 with a similarly positively-charged lysine also resulted in complete inhibition of binding (Fig. 3, p. 1279) (11). Alanine substitution at Q23 on the B strand had a

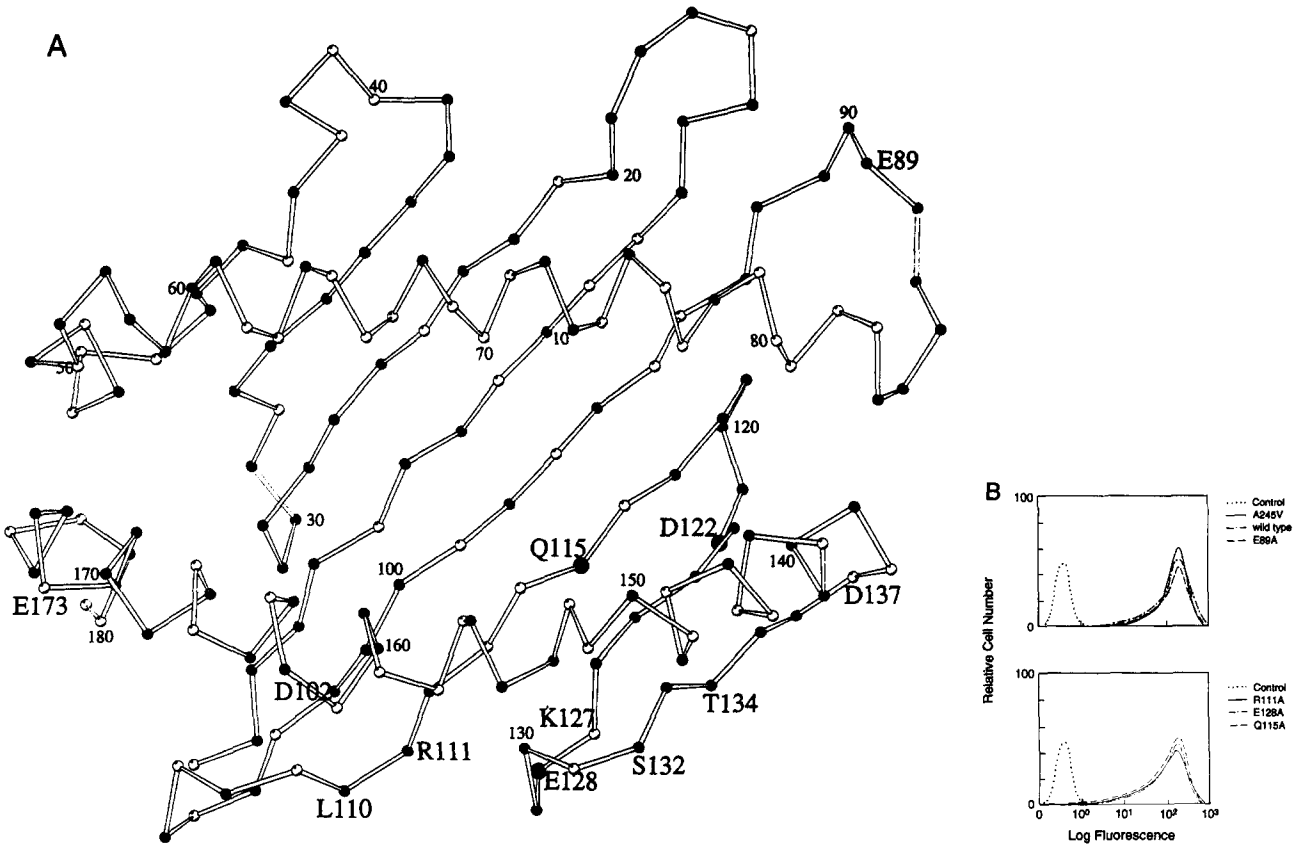
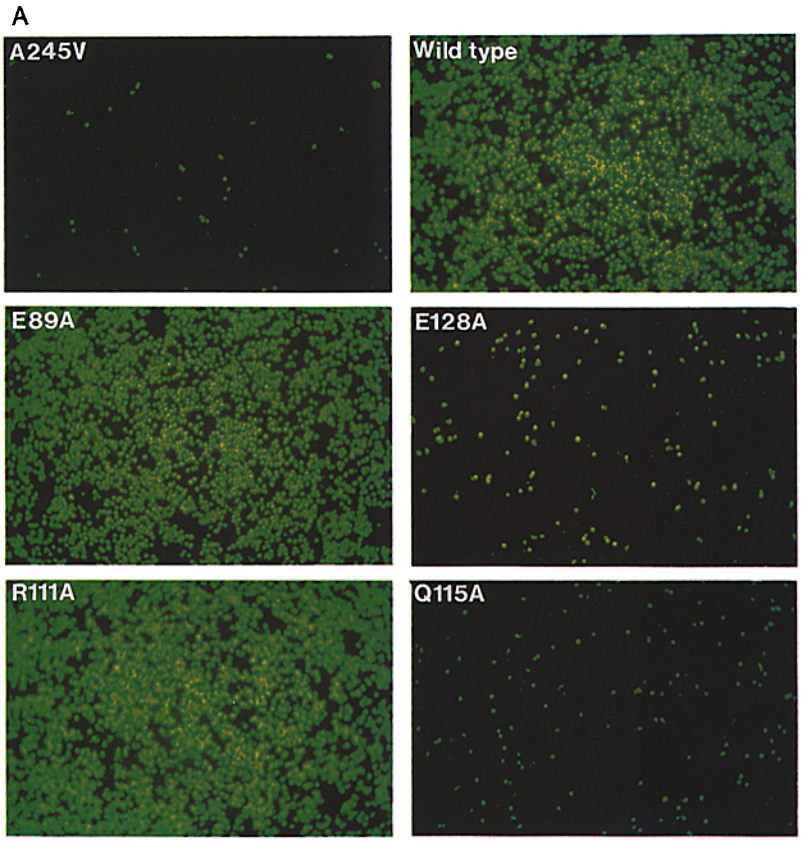


Figure 1. Positions of MHC class I variants and expression on C1R cell transfectants. (*a*) α-carbon backbone of the α1 and α2 domains of class I (HLA-A2). The domains and the peptide-binding groove are shown as viewed from the top of the molecule. Closed circles indicate conserved residues, and open circles nonconserved. The figure is modified from Fig. 4 of reference number (37). (*b*) Stable expression of wild-type and mutant HLA-A2 molecules in C1R cells. Shown are representative flow cytometry profiles from one experiment. Expression levels were monitored in every assay, and samples were stained with HLA-A2–specific mAb MA 2.1 (18) plus fluoresceinated goat anti–mouse IgG as described (6).

similar effect, suggesting that this residue may participate in the interaction as part of the R4/L25 surface of CD8. The effect of mutations in other positions on binding ranged from moderately-affected (L8, E19, and T47) to unaffected (D9 and H106). It appeared that only a small number of residues on the CD8 A and B but not the G β-strands were critical to the interaction with the α2 domain of MHC class I.

Examination of the crystal structure of HLA-A2 reveals a large open cavity on the molecular surface (Fig. 4, *a* and *b*). The cavity is composed of the α1α2 platform, the loops of the α3 domain and a part of β_2m with the CD8 contact residues all on one side of this cavity. About one-third of the underside of the α1α2 β-sheet constituting the floor of the groove is exposed to this space (13). Residues Q115 and D122 are both located on the floor of the peptide-binding groove with their side chains pointing down towards this cavity (Fig. 4 *a*). Residues Q115, D122 and E128 are completely conserved in 22 human (24, 25) and 12 murine (26) sequences. Our results are supported by the observation that murine primary $CD8^+$ T cells responded more vigorously to an HLA-A2 hybrid with the murine α2α3 domain than with only the murine α3 domain (27). The impact of the α2 domain of class I on recognition by $CD8^+$ T cells is further supported by a recent study in which CTL responses in human CD8 transgenic mice to an $A2/k^b$ hybrid molecule (human α1α2 domain, murine α3 domain) were markedly enhanced compared to responses in nontransgenic mice. These results provide functional evidence for a second interaction site outside of the α3 domain that is essential for optimal coreceptor function (28). In experiments discounting the involvement of the α2 domain in binding CD8, most positions tested for binding were either polymorphic or on the upper faces of the α1α2 domains (5, 6). Interestingly, two of the three critical residues in the α2 domain of class I found in this study were negatively charged whereas residue R4 on the lateral side of CD8 was positively charged. This charge complementarity coincides with that of the contact surface between the α3 loop of class I and the CDR-like regions of CD8, supporting a crucial role for electrostatic interactions between CD8 and class I molecules (6, 11).

Previous work on the interaction between CD8 and MHC class I had implicated the importance of the CDR-like loops of CD8 and the α3 domain of class I (5, 20). Our results extend this interaction to include the α2 domain of class I interacting with the A/B surface of CD8. A CD8α/α homodimer would fit into the class I/β_2m cavity through



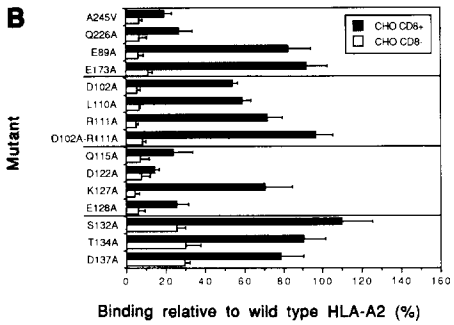
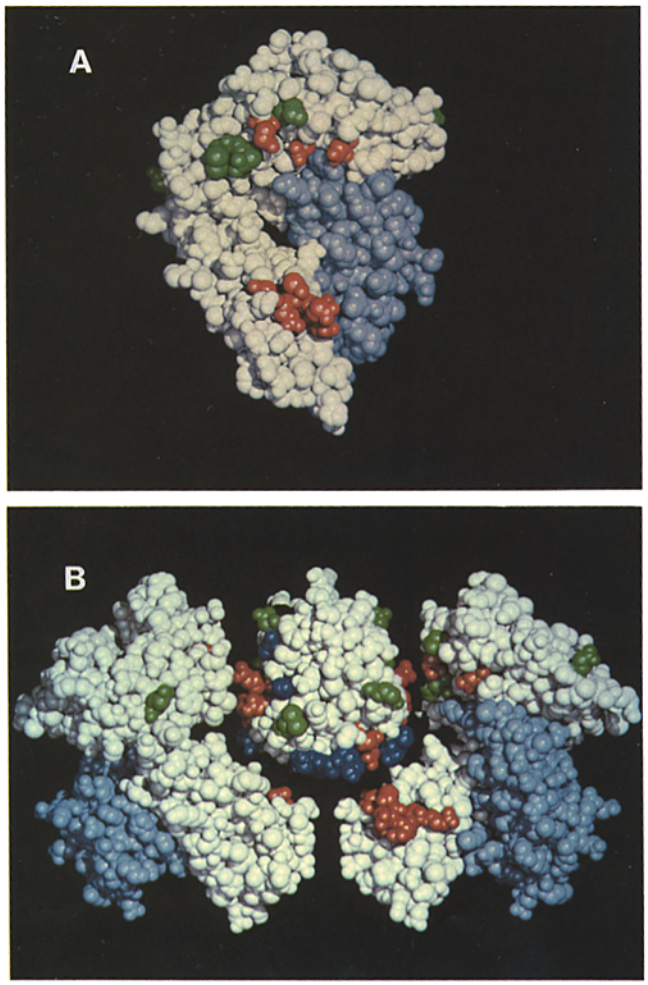


Figure 2. Cell–cell adhesion assay determining binding of MHC class I to CD8α. (*a*) Fluorescent microscopy of labeled C1R cells expressing wild-type or mutant class I variants bound to a monolayer of $CD8^+$ CHO cells (MT8.02) (12). (*b*) Adhesion of C1R cell transfectants expressing mutant class I to either CD8+ (closed, MT8.02 line) or CD8− CHO cells (open, MT8.C13 line) (12). A2 transfectants were grouped based on their locations on the surface of class I molecule. Results from three experiments were averaged (E173A from two experiments) and were expressed relative to wild-type binding. S132, T134, and D137 transfected C1R cells were grown in RPMI 1640 tissue culture medium. Error bars represent SE. For each mutant, 2-6 repeated assays gave similar results. The binding assay was conducted in double blind fashion. D129A showed a significant loss of binding (15–20% of wild type), but due to its noticeable loss of expression level on C1R cells (75% mean fluorescence intensity of wild type), results were not included in the figure.



part of its CDR-like loops and the entire A/B binding surface from one monomer (Fig. 4 *a*). Given this alignment, one CD8α/α homodimer could interact simultaneously with two symmetrically related HLA molecules (Fig. 4 *b*). Our results provide support for this model in that the three critical residues in the α2 domain were predicted to interact with CD8 based on the model. Assuming the model is correct, there are residues on β_2m that are likely to interact with CD8 as well.

The alignment of CD8 with class I has important implications for the function of the CD8α/α molecule. CD8 is

Figure 4. Models of mutations located on the CD8α/α homodimer and MHC class I molecules affecting interaction between the two molecules. Models were generated by Quanta (Polygen, Waltham, MA). (*a*) The class I molecule is shown as viewed perpendicularly to the pseudo-dyad axis of the α1α2 domains. The α1 and α2 domains are at the top with the α2 helices in front. Directly underneath α1α2 is the β_2m subunit (*light blue*). Positions where substitution to an alanine led to complete inhibition of binding are in red (Q115, D122, E128, and the 223–229 loop). Amino acids where substitutions had minimal effects on binding are in green (E89, D102, L110, R111, K127, and E173). The open cavity on the surface of the MHC class I crystal is composed of the α1α2 platform, the loops of the α3 domain and a part of β_2m. Another angle of this cavity is revealed in the right side view of MHC classs I in *b*. (*b*) Potential interactions between a single CD8α/α homodimer and two MHC class I molecules. CD8 is rotated 90° about its dimer axis and the CDR-like loops are facing down. The left and the right flanking MHC class I molecules are viewed perpendicularly to the α1α2 pseudo-dyad with the cleft viewed end- and head-on, respectively. The molecules have been pulled apart to better display the interaction. Positions on CD8 that led to complete inhibition or had minimal effects with alanine substitutions are in red and green, respectively. Positions on CD8 that led to inhibition with only the charge/size substitution are in dark blue (11).

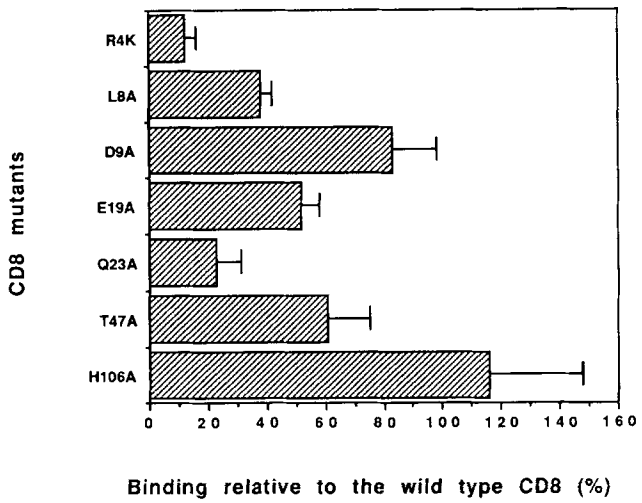


Figure 3. Cell–cell adhesion assay of mutant forms of CD8α to MHC class I. Shown are data from three experiments expressed relative to wild-type binding. Error bars represent SE. A, B, C′ and G β-strands of CD8α span residues 3–12, 17–24, 45–51, and 102–113, respectively (10).

likely to induce dimerization of MHC class I. It has been shown that cross-linking class I led to signal transduction (29–32). Conversely, the dimerization may bridge two TCRs interacting with class I molecules, and thus promote the capping of TCR and facilitate complex formation between TCR and associated signal molecules. Such a model was proposed for T cell receptor, CD4, and MHC class II complexes based on the finding that the crystals of MHC class II molecules were dimers of dimers (33).

The CD8 molecule exists as either an α/α homodimer or α/β heterodimer. Only the CD8α/α homodimer is expressed on a subset of human NK cells and T cell receptor γ/δ cells in the gut. Both forms are present on thymocytes and peripheral T cells, however, the α/β heterodimer is predominant. The heterodimer could also be bivalent by analogy with growth hormone and its receptor (23). Growth hormone interacts through two nonidentical binding sites to nearly identical sites on two hormone receptors.

Another potential consequence of CD8 interaction with the α2 domain could be to influence the conformation of MHC peptide formed by the α1 and α2 domains. Small conformation effects might affect TCR-peptide-MHC interactions. It has been shown that small changes in peptides can significantly alter the outcome for TCR activation through differential TCR signaling (34, 35). The requirement for CD8 in TCR triggering has generally been thought to result from increases in TCR-peptide-MHC avidity by interaction of CD8 with the α3 domain. Supporting the notion that CD8 may be doing more than just increasing avidity solely through interaction with MHC, a recent paper demonstrated a role for CD8-TCR interaction in modulating TCR–ligand interaction (36). Our work suggests the possibility of modulation of TCR–ligand interaction through CD8-class I α2 domain interaction.

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Address correspondence to Dr. Paula Kavathas, Fitkin 617, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06520-8035.

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