Mutations in CD8 that Affect Interactions with HLA Class I and Monoclonal Anti-CD8 Antibodies

By S. K. Sanders,* R. O. Fox,§ and P. Kavathas*‡

*From the Departments of *Laboratory Medicine, Genetics, and ‡Immunobiology, Yale University, New Haven, Connecticut 06510; and the §Department of Molecular Biophysics and Biochemistry and the Howard Hughes Medical Institute, Yale University, New Haven, Connecticut 06511*

Summary

The T cell co-receptor, CD8, binds to the α3 domain of HLA class I (Salter, R.D., R.J. Benjamin, P.K. Wesley, S.E. Buxton, T.P.J. Garrett, C. Clayberger, A.M. Krensky, A.M. Norman, D.R. Littman, and P. Parham. 1990. *Nature [Lond.]* 345:41; Connolly, J.M., T.A. Potter, E.M. Wormstall, and T.H. Hansen. 1988. *J. Exp. Med.* 168:325; and Potter, T.A., T.V. Rajan, R.F. Dick II, and J.A. Bluestone. 1989. *Nature [Lond.]* 337:73). To identify regions of CD8 that are important for binding to HLA class I, we performed a mutational analysis of the CD8 molecule in the immunoglobulin (Ig)-like variable domain. Our mutational analysis was based on our finding that using a cell-cell adhesion assay murine CD8 (Lyt-2) did not bind to human class I. Since the interaction of human CD8 with HLA class I is species specific, we substituted nonconservative amino acids from mouse CD8 and analyzed the ability of the mutated CD8 molecules expressed in COS 7 cells to bind HLA class I-bearing B lymphoblastoid cells, UC. Mutants with the greatest effect on binding were located in a portion of the molecule homologous to the first and second hypervariable regions of an antibody combining site. In addition, a panel of 12 anti-CD8 monoclonal antibodies were used to stain the 10 CD8 mutants, and amino acids that affected antibody binding were localized on the crystal structure of the Bence-Jones homodimer, REI. Support for an Ig-like structure of CD8 can be found in the pattern of substitutions affecting antibody binding. This work supports the similar tertiary structure of the CD8 α-terminal domain and an Ig variable domain.

The TCR-CD3 complex associates with CD8 or CD4 to form a multimeric complex interacting with peptide and HLA class I or HLA class II, respectively, during T cell activation. In addition to their function as adhesion molecules, CD8 and CD4 also serve as signalling molecules. The cytoplasmic domains of CD4 and CD8α bind to the NH_2-terminal domain of the intracellular tyrosine protein kinase $p56^{lck}$ (1–3). Antibody crosslinking of CD8 and CD4 to the TCR-CD3 complex leads to enhanced calcium flux and phosphorylation of a set of proteins, which includes the ζ chain of the TCR complex (4–6). The ability of CD8 to induce these changes is dependent on its ability to associate with $p56^{lck}$. CD8 and CD4 are also important for positive and negative selection in the thymus (7, 8). TCR specificity in T cell repertoire selection is determined by both the TCR-α/β heterodimer and CD4 or CD8 accessory molecules (9, 10).

The human CD8 molecule is expressed either as an α/α homodimer or as an α/β heterodimer. The homodimer is expressed exclusively on subsets of TCR-γ/δ cells and NK cells, and is co-expressed with CD8β on peripheral T cells and thymocytes. Individual human peripheral T cells can express varying amounts of CD8 α/α and α/β complexes, and these appear to be differentially regulated upon T cell activation (11). CD8 expression can be induced on human CD4 cells upon activation with mitogens (12) and on murine CD4 cells cultured in IL-4-containing medium (13). Con A-activated γ/δ T cells that are $CD4^-8^-$ can be induced to express the CD8 homodimer on a subset of cells. Thus, CD8 is differentially expressed during development, cell activation, and in lymphocyte subsets.

Direct binding of CD8 to HLA class I and CD4 to HLA class II was shown using cell-cell adhesion assays in which CD8 or CD4 were expressed at high levels on the surface of chinese hamster ovary (CHO) cells for CD8 (14) or monkey CV1 or COS 1 cells for CD4 (15, 16). Purified class I and CD8 molecules expressed in lipid-sealed spherical nylon vesicles also showed specific binding (17). The affinity of interaction of CD8 with MHC class I is increased upon activation through the TCR (18). Murine CTL were able to bind to an H-2 molecule on a planar membrane not recognized by the TCR only after T cell activation (18). Thus, CD8 can bind to class I in the absence of a TCR stimulus only when the levels of CD8 are high.

Mutations or natural polymorphisms in the α3 domain of HLA class I affect binding of CD8 to HLA class I (19–21). By mutational analysis, Salter et al. (19) found that a highly conserved exposed loop involving residues 223–229 of the α3 domain of HLA class I was important for binding. This was considered a minimal CD8-binding site since two other clusters of amino acids at positions 233 and 235, and positions 245 and 247 in the α3 domain of HLA class I, were also important for binding. The latter positions 245 and 247 were buried in the molecule and therefore considered unlikely to be direct contact points (19). In the mouse, residue 227 of H-2 class I molecules was shown to be important for recognition by CD8-dependent, but not CD8-independent, CTL, implicating its role as part of a determinant that is the ligand of the CD8 molecule (21).

In this report, we performed a mutational analysis of the CD8α molecule to determine what regions or amino acids were important for binding to HLA class I and for anti-CD8 antibodies. We identified two regions of the CD8 molecule that are important for the species-specific interaction of CD8α with HLA class I. These regions correspond to the CDR1 and CDR2 hypervariable regions of immunoglobulin REI (22), known as the complementarity determining regions (CDR). These regions are not conserved between mouse and human CD8. Our work supports the hypothesis that there may be additional binding sites in the α3 domain of HLA class I besides the conserved loop (residues 223–229) that would be important for species-specific CD8-HLA class I interaction.

Materials and Methods

DNA Constructs and Mutagenesis. Human CD8α cDNA (23) was subcloned into the HindIII-BamHI site of Bluescript SK^+. Mouse CD8α was cut out of the PM7-CD8L vector (24) with XbaI and PstI and subcloned into the BamHI-PstI sites of Bluescript SK^+. Human and mouse CD8 were excised from the Bluescript vector with BamHI-HindIII, and HindIII-Xba I, respectively. The inserts were blunted with the Klenow fragment of DNA polymerase I, ligated to EcoRI adaptors (New England Biolabs, Beverly, MA), and subcloned into the EcoRI site of the pCDL-SRα296 vectors (25). CD8 mutants M1-M10 were generated by oligonucleotide-directed mutagenesis with single-stranded phagemid DNA (26).

Transfection of Cos 7 Cells. Cos 7 monkey kidney cells (27) were transfected with SRα296 constructs (human CD8 wild-type and CD8 mutants M1-M10, mouse CD8 [Lyt-2], and vector alone) as follows: 3×10^5 cells plated 24 h earlier in 35-mm Primaria tissue culture dishes (Falcon Labware, Oxnard, CA) were transfected with 5 μg plasmid DNA (CsCl-banded twice) in 1.0 ml OPTIMEM plus 25 μl lipofectin (1 mg/ml) (Gibco-BRL, Gaithersburg, MD) (28). Lipofectin remained on the COS 7 cells for 18 h. Transfected COS 7 cells were used 48 h later in the cell-cell adhesion assay described below.

CD8-HLA Class I Cell-Cell Adhesion Assay. Transfected COS 7 cells were washed once with PBS and 10^7 ^{35}S-cysteine-labeled B cells, UC (29), were added to each 35-mm dish in 1.0 ml PBS supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin-streptomycin. The cells were incubated for 1 h at 37°C and the B cells were poured off. The dishes were washed seven times by adding 1 ml PBS containing FCS and plunking each dish from a height of 3 inches. After washing the unbound B cells from each dish, 0.6 ml PBS containing 1% NP-40 was added to each well. Cells were incubated 20 min on ice, the NP-40 lysate was removed, and 50 μl was placed in aqueous scintillation flour and counted to determine the number of B cells bound to the transfected COS 7 cells.

^{35}S-Cysteine Labeling of B Cells. 10^8 B cells were labeled with 1 mCi ^{35}S-cysteine (Amersham Corp., Arlington Heights, Ill) for 2 h at 37°C in cysteine-free RPMI containing 10% dialyzed FCS, washed two times, and then used for binding as described above. Approximately 50% of the ^{35}S-cysteine was incorporated into the UC cells in 2 h, and the specific activity ranged from 7 to 12 cpm per B cell.

Anti-CD8 Monoclonal Antibodies. A panel of 12 anti-CD8 mAbs were used to stain COS 7 cells transfected with the mutant CD8 molecules M1-M10. The 10 mouse mAbs used were OKT8 (30), 51.1, 66.2, G17.5, G19.5, and G10.1 (obtained from J. Ledbetter, Bristol-Myers Squibb Research Institute, Seattle, WA), and Leu-2a, Leu-2b, Leu-2c, and Leu-2d (Becton Dickinson & Co., San Jose, CA). The three rat anti-CD8 mAbs were Campath B and Campath 8B, provided by H. Waldman (Cambridge University), and the anti-murine CD8 antibody 53.6 (31).

FACS® Analysis of Transfected COS 7 Cells. For FACS® analysis, transfected COS 7 cells were removed from the dish with PBS containing 0.6 mM EDTA, stained with saturating concentrations of anti-CD8 mAbs as described (32), and analyzed after staining with either a 1:50 or 1:25 dilution of FITC-conjugated goat anti-mouse IgG or goat anti-rat IgG (Southern Biotechnology, Birmingham, Alabama) on a FACScan® (Becton-Dickinson & Co.). Propidium iodide was included to gate out dead cells. The FITC-conjugated goat anti-mouse IgG or the goat anti-rat IgG served as a negative control.

Analysis of Cell-Cell Adhesion Assay. Since the cell-cell adhesion assay is transient, it was necessary to interpret the binding not only in relationship to wild-type binding on that day, but also to take into account the mean fluorescence intensities of the OKT8 antibody staining of mutants M1-M7 and M9 or G19.5 antibody staining for mutants M8 and M10 and the respective wild-type antibody staining on that day. Therefore, the binding of UC cells to COS 7 cells transfected with CD8 mutants was corrected for binding of UC cells to COS 7 cells transfected with vector alone and was normalized for the linear mean fluorescence intensity (fl) of COS 7 cells stained with mAb OKT8 (for mutants M1-7 and M9) or mAb antibody G19.5 (for mutants M8 and M10) and for the binding obtained with wild-type CD8. We used the following formula (33): 100 × % binding = [(mutant binding − vector binding)/mutant OKT8 or G19.5 fl]/[(wild type binding − vector binding)/wild type OKT8 or G19.5 fl].

Results

Because the least homology between human and mouse CD8 exists in the Ig-like domain (42% vs. 64–80% for the cytoplasmic and transmembrane regions, respectively), we hypothesized that this difference could lead to differences in ability of these two molecules to bind to HLA class I. We therefore transfected cDNA for either mouse or human CD8 into COS 7 cells and determined whether these cells could bind the HLA class I–expressing human B lymphoblastoid cell line, UC, (29). Detectable binding of UC cells to human CD8 expressing COS 7 cells could be observed using phase contrast microscopy (Fig. 1 *a*), whereas minimal binding of

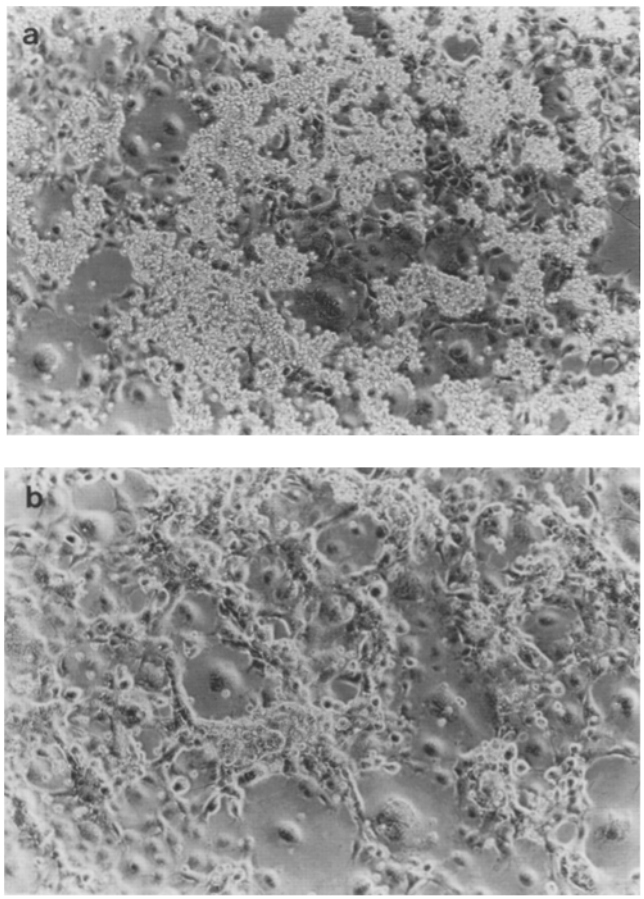

Figure 1. Differences between mouse and human CD8 in binding to MHC class I–expressing B cells. Phase contrast microscopy of small, UC, B cells bound to a monolayer of COS 7 cells transfected with human CD8 (*a*) or mouse CD8 (*b*) (×100).

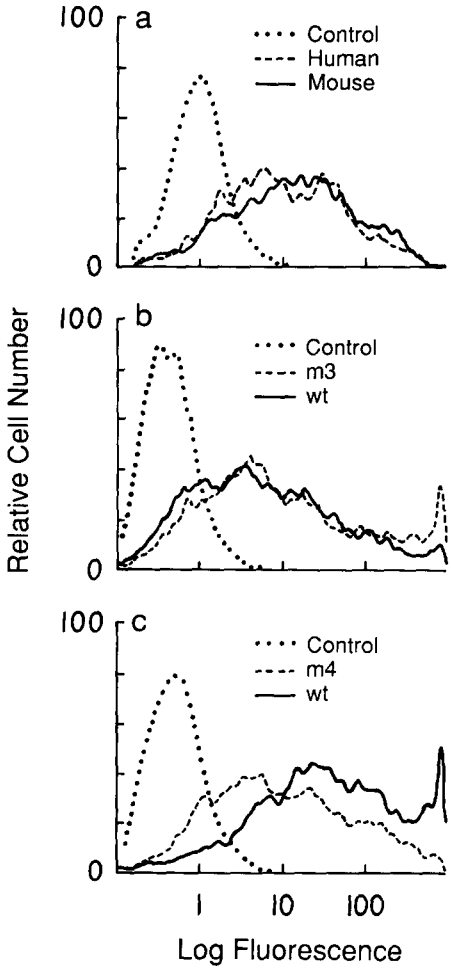

Figure 2. FACS® assayed CD8 expression (*a–c*) of COS 7 cells transfected with murine or human CD8 (*a*), and CD8 mutants (*b* and *c*). (*A*) FACS® histogram of COS 7 cells transfected with murine CD8, stained with rat mAb 53.6 (31), or wild type human CD8, stained with mouse mAb, OKT8 (30), followed by second-step staining with FITC-conjugated goat anti-rat IgG or goat anti-mouse IgG. Control cells are stained with second-step antibody alone. (*b*) FACS® histogram of OKT8 antibody binding to COS 7 cells transfected with wild-type human CD8 or mutant M3. The second-step reagent was FITC-conjugated goat anti-mouse IgG. Control cells are stained with second-step antibody alone. (*c*) FACS® histogram of OKT8 antibody binding to COS 7 cells transfected with wild-type human CD8 or mutant M4 stained as described above in *b*.

UC cells to COS 7 cells expressing mouse CD8 (Fig. 1 *b*) or vector alone transfected COS 7 cells was observed (data not shown). This was not due to differences in expression levels, because COS 7 cells expressing human and mouse CD8 had similar mean fluorescence intensities after staining with anti-CD8 antibodies (29 vs. 31 arbitrary units, respectively; Fig. 2 *a*).

Since mouse CD8 did not bind human class I, we reasoned that differences in amino acids in the variable-like region could be important. Thus, we made a panel of 10 mutants by substituting nonconservative amino acids from mouse CD8 into the human CD8 molecule from cysteine 22 to 94 (Fig. 3), a portion of the variable-like region of CD8 that is most likely to resemble an Ig fold (34). The mutants were transfected into COS 7 cells and analyzed for binding to ^{35}S-cysteine-labeled B cells and a panel of 12 anti-CD8 mAbs.

CD8 mutants were grouped based on their effect on binding to HLA class I–expressing B cells, UC (Fig. 4). Group 1 (M2) exhibited little or no binding of class I–expressing UC cells. The percent relative wild-type binding was within the range (−0.6–5.8%) of binding observed for mouse CD8-transfected COS 7 cells. Group 2 (M1, M3, M5) bound low amounts of HLA class I–expressing UC cells (9–16% of wild type). Group 3 (M6, M7, M9, M10) bound intermediate amounts of class I–expressing UC cells (31–57% of wild type). Group 4 (M4, M8) bound similarly to wild type (>80% of wild type). The percent relative binding was calculated with a correction for the level of CD8 expression after transfection into the COS 7 cells since this variable has a direct effect on binding (17). However, even without this correction, the mutants fell into similar groupings, except for M4, which would have fallen into group 3.

Mutant M2 (group 1) is a point mutant (leucine 26 to glycine) that totally eliminated binding of the class I–expressing UC cells relative to wild type (1.7% ± 1.02; Fig. 4). Mutant M2 was always expressed equal to or better than wild-type, and the OKT8 mAb staining profile was similar to that

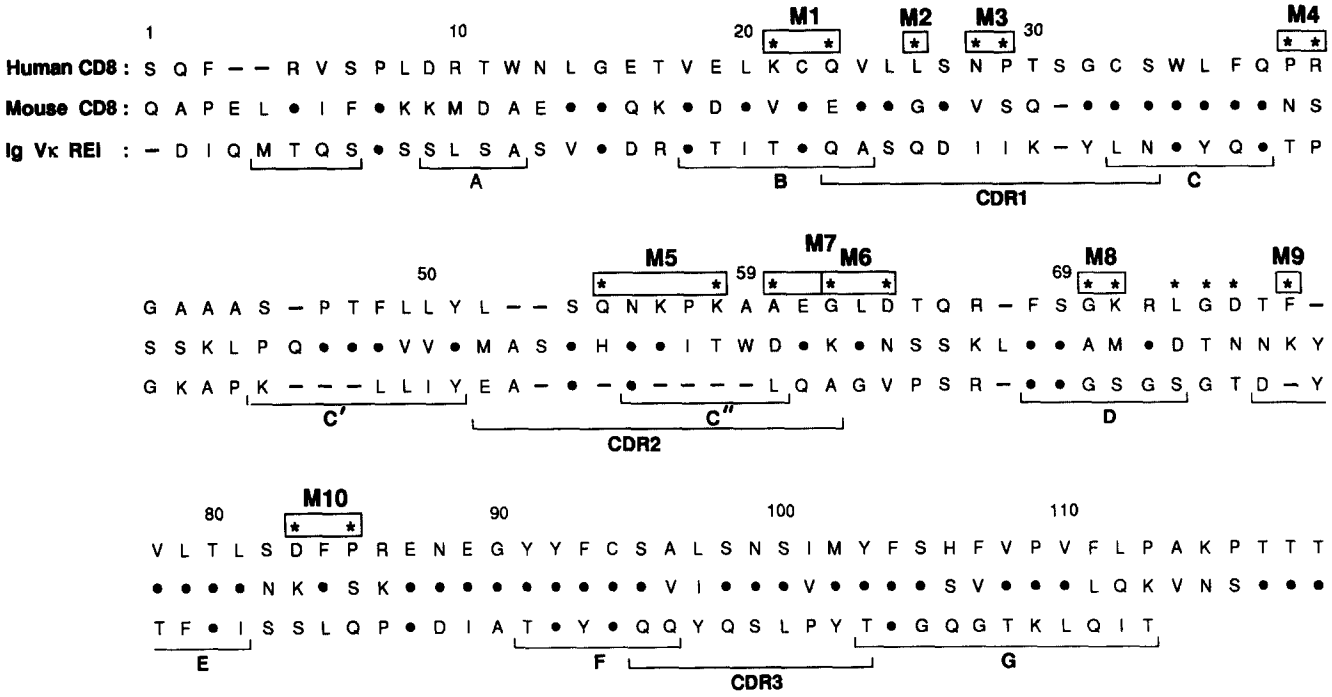

Figure 3. Sequence alignment of human and murine CD8 with the Ig variable κ light chain protein, REI. The top line shows the amino acid sequence of the Ig-like variable region of human CD8. The middle line shows the corresponding sequence of murine CD8 (48). The bottom line shows the variable region sequence of the Bence Jones protein REI (37). Numbers indicate the amino acid number of the human CD8 protein. Asterisks indicate nonconservative amino acid changes between human and murine CD8. Boxes indicate positions of mutants, M1-M10, from left to right, except M6, which changes the last two of the three amino acids changed in M7. The arrows under the REI sequence indicate the β strands of REI as determined from the crystal structure (37). CDR1, CDR2, and CDR3 correspond to the Ig hypervariable regions (22). Dots indicate positions where murine CD8 or REI are identical to human CD8. The sequence alignment requires relatively few insertions or deletions of residues with the exception of a large insertion in the CDR2 and β strand C″ regions of CD8 making this region of the alignment the most uncertain.

of mutant M3 shown in Fig. 2 *b*. Since mutant M2 was expressed as well as wild-type CD8 and was a point mutant that did not affect the staining of a panel of 12 anti-CD8 mAbs, we predict that mutant M2, which resides in the CDR1

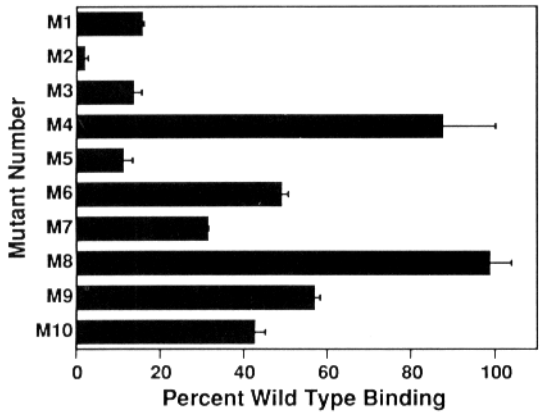

Figure 4. Binding of mutants to HLA class I–expressing B cells, UC, relative to wild-type binding. Error bars represent SEM. There were two to three experiments for each mutant. Between 9.4 and 15.4% of the UC cells bound to wild-type human CD8-transfected COS 7 cells, which was 4.2–9.2-fold above UC cells bound to vector alone. UC cells bound to murine transfected COS 7 cells ranged from −0.6 to 5.8% compared to wild-type. Human CD8, murine CD8, and vector alone transfected COS 7 cells were included in each of seven experiments.

hypervariable-like region of Ig (22), is either defining a contact point or is creating local conformational distortion.

Mutants M1, M3, and M5 (group 2), exhibited low amounts of binding to class I–expressing UC cells (M1 [15.65 ± 0.53], M3 [13.5 ± 2.09], M5 [11 ± 2.4]; Fig. 4) relative to wild type. All three mutants in this group were expressed equal to or better than wild type. Like mutant M2, mutant M5 bound all antibodies well. Mutant M5 has two amino acid changes (glutamine 54 to histidine and lysine 58 to threonine) located in the CDR2-like region. CD8 has many more amino acids in this region compared with immunoglobulin REI, so that if CD8 has an Ig-like structure, the L2 loop is likely to be larger as compared with Ig. Mutant M1 has two amino acid changes flanking cysteine 22 (lysine 21 to valine and glutamic acid 23 to glutamine), which are located in β-strand B and CDR1. This cysteine is likely to disulfide bond to cysteine 33 since in mouse CD8 the cysteines at position 26 and 36 form a disulphide bond compatible with an Ig fold (35). Mutant M1 affected binding of anti-CD8 mAbs Leu-2c and Campath 8B (2% and 22.0%, respectively, relative to wild-type staining; Table 1). Because the amino acid changes in mutant M1 flank cysteine 22, which is likely to be involved in an intrachain disulfide bond and has two anti-CD8 antibodies that are severely affected in their staining, the total conformation of the CD8 molecule may have been affected by this mutation. Mutant M3 resides in the CDR1 region and has two amino acid changes (asparagine 28 to va-

Table 1. *CD8 Antibody Staining Affected by CD8 Mutations*

Mutant number	Percent relative linear mean fluorescence intensity of CD8 antibody staining						
	66.2	G17.5	Leu-2a	Leu-2b	Leu-2c	Leu-2d	Cam8B
M1	–	–	–	–	2.0	–	22.0
M3	3.0	40.0	–	–	–	–	–
M6	–	–	–	–	–	4.0	–
M7	–	–	–	–	–	4.0	–
M8	–	–	4.0	–	–	–	–
M9	–	22.0	–	–	–	–	–
M10	–	–	–	1.2	–	1.9	–

A panel of 12 CD8 mAb was used to stain wild-type and mutant CD8 transsfected COS 7 cells. The 10 mouse mAbs were OKT8, 51.1, 66.2, G10.1, G17.1, G19.5, Leu-2a, Leu-2b, Leu-2c, and Leu-2d. The two rat mAbs were campath B and campath 8B. Dashes indicate that the antibody staining was equivalent to wild type. Mutants with mAb staining <50% of wild type are indicated by numbers.

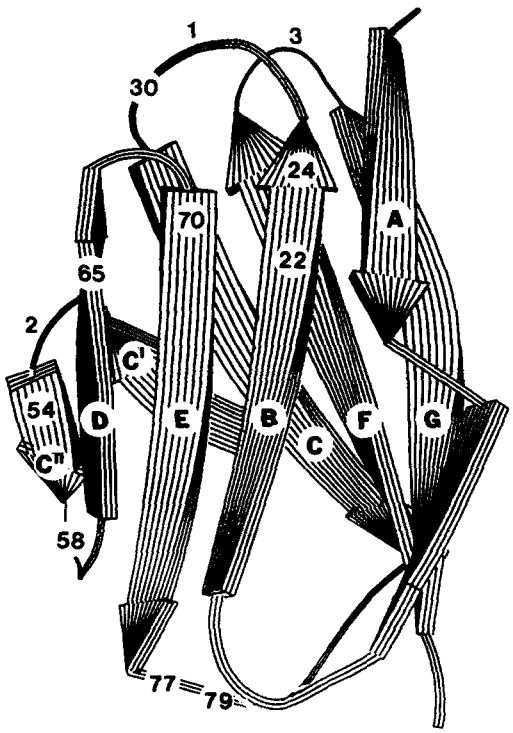

Figure 5. Location of residues in the variable region of CD8 affecting binding of anti-CD8 antibodies on the α-carbon skeleton derived from coordinates of the Vκ Bence-Jones homodimer, REI (37). The diagram was generated using the program RIBBON (49). One member of the dimer is shown. Numbers *1*, *2*, and *3* on the diagram refer to the hypervariable loops L1, L2, and L3 (36), and the letters *A*–*G* indicate the β strands. The sheet forming the V_L dimer interface is distant from the viewer. The numbered positions indicate REI residues corresponding to the amino acid substitutions in mutants M1 (22, 24), M3 (29, 30), M6/7 (54, 56, 58), M8 (64, 65), M9 (between 70 and 71), and M10 (77, 79), which affected the following anti-CD8 mAbs, respectively (see Table *I*): Leu-2c and cam8B, 66.2 and G17.5; Leu-2d, Leu-2a, G17.5; Leu-2b and Leu-2d. There is no corresponding REI residue for mutant M9 (see Fig. 3), therefore, REI residue 70 located on one side of the mutant is labeled. The biggest discrepancy between REI and CD8 is in the L2 and β strand C" due to a large insertion in CD8. It is likely that to accommodate the extra amino acids in CD8, the L2 loop would be more extensive and would project towards the other hypervariable loops L1 and L3.

line and serine 29 to proline, respectively). The OKT8 mAb staining profile for mutant M3 is shown in Fig. 2 *b*. Mutant M3 abolished mAb 66.2 binding and reduced G17.5 mAb binding to 40% of wild type. Thus, mutants exhibiting low amounts of binding reside in hypervariable-like regions CDR1 and CDR2.

Mutants M6, M7, M9, and M10 (group 3) exhibited intermediate amounts of binding of class I–expressing UC cells (M6 [49.1 ± 1.73], M7 [31.3 ± 0.28], M9 [56.9 ± 1.43], M10 [42.6 ± 2.58]; Fig. 4). In this group, only mutant M7 was expressed as well as wild type, whereas mutants M6, M9, and M10 were never expressed as well. For this reason, we cannot be confident of the exact numbers obtained in the binding assay. All we can say is that they do not exhibit low amounts of binding to class I–expressing UC cells. Mutant M6 changes two amino acids (glycine 62 to lysine and aspartic acid 65 to asparagine) located in CDR2 and just outside. Mutant M7 has three amino acid changes, the same two as M6 with one additional amino acid change (alanine 60 to aspartic acid). Both mutants M6 and M7 abolished Leu-2d mAb binding (Table 1). Because mutant M7 has one additional amino acid change at position 60 compared to mutant M6, which has changes at positions 62 and 64, and exhibits 31% binding compared to 49% for M6, this change from an aspartic acid to an alanine may be more important for binding than amino acids 62 and 64. Alternatively, all three amino acids may have an additive effect on binding. The binding of mutant M9 (phenylalanine 77 to asparagine; Fig. 3) was 57% of wild type, which could either be due to decreased expression or because of its potential contact with loop 1 (36) (see below). Mutant 10, which had two amino acid changes (aspartic acid 83 to lysine and proline 85 to serine, respectively) bound 43% relative to wild type and was negative for binding of the two antibody epitopes, Leu-2b and Leu-2d. It was also partially affected for OKT8 mAb staining (70% of wild type). The multiple effects on antibody binding could reflect overlapping epitopes or a disruption of overall stability.

Mutants M4 (proline 39 to asparagine and arginine 40 to serine) and M8 (glycine 70 to alanine and lysine 71 to methionine) were not affected in their binding to class I–expressing UC cells (Fig. 4). Mutants M4 and M8 were never expressed as well as wild type. An example of the OKT8 mAb staining profile for mutant M4 is shown in Fig. 2 *c*. Of these two mutants, only M8 did not bind all mAbs well. Mutant M8 destroyed Leu-2a mAb binding (Table 1).

The protein sequence of CD8 was aligned with that of the REI light chain using conserved IgG residues as a guide. A molecular model for the CD8 α/α homodimer was generated using the REI light chain dimer crystal structure as a

three-dimensional template (37). Side chain substitutions were made using the graphics program INSIGHT II, and internal packing of the structure was defined and analyzed using the program PICOPAK (38). Despite the low level of sequence identity, the internally packed residues were replaced by like hydrophobic side chains leading to an efficient repacking of the protein interior. Small repositioning of the backbone was required to accommodate general internal side chain substitutions. Amino acid substitutions at the REI light chain dimer interface were also conservative resulting in a well-packed extended surface suggesting that this mode of interaction is a valid model for the CD8 α/α homodimer interface.

Based on modelling with REI, we located the relative positions of amino acid changes that affected antibody binding. In most cases, antibody binding was affected by a single mutant, whereas in two cases, mutants in different locations were affected. All amino acid positions affecting antibody binding were available to the solvent and presumably could bind antibodies (Fig. 5). Mutants M3 and M9 both reduced G17.5 antibody staining to 40 and 22%, respectively, relative to wild type. Mutant M3 is located in CDR1 and corresponds to REI positions 29 and 30, whereas mutant M9 is either in the loop between β strands DE or located in β strand E (Fig. 3). These two regions may be in close proximity if the loop extends into the loop between β strands B and C (Fig. 5). Mutants M6, M7, and M10 all affected Leu-2d antibody staining. Mutants M6 and M7 are more difficult to position because of the extra amino acids in CD8 compared with REI in this region. Given this caveat, their position either in β strand C″ or between β strands C″D is spatially close to the position of mutant M10 lying between β strands EF. Thus, the location of mutants affecting the same mAb that are spatially distant on a linear map are spatially close on a three-dimensional model of CD8 based on Ig structure. These results support the hypothesis that the CD8 structure is similar to Ig.

Discussion

A mutational analysis of amino acids in CD4 important for MHC class II binding has also been reported (16, 39). Mutations in the CDR1- and CDR2-like regions as well as outside the predicted CDR loops affected binding (16). However, the strongest effects were observed for mutations in the CC′ and C″D corners of domain 1 and along the A strand of domain 2 of the four Ig-like domains in CD4. This led to the hypothesis that the CD4-MHC class II interaction would be similar to Ig but not exclusively Ig like. A genetic analysis of mAb and HIV binding sites on CD4 also affirmed the similar tertiary structures of the CD4 NH_2-terminal domain and Ig variable regions (40). Our results on mAb binding and binding to MHC class I for CD8 are best explained by invoking an Ig-like structure for CD8 as well.

After completion of this work, two reports on the crystallization of the outer two domains of CD4 were published (41, 42). In this very exciting work, the Ig-like structure of both domains was established. Domain 1 (98 amino acids), which is similar in size to an Ig domain, has nine β strands folded like a "sandwich" with a hydrophobic core. The major differences with Ig lie in the loops. For instance, the turn between strands CC′ and FG is shorter. These regions are thought to be important in the dimer formation of an Ig molecule, and the FG loop contains the CDR3-like region important for antigen binding. These regions would presumably not be necessary for CD4, which is a monomer. Because CD4 mutations in the CC′ and C″D corners of domain 1 along with the A strand of domain 2 had the strongest effect on binding, it was proposed that the two domains may form a notch for interlocking CD4 with MHC class II (41).

The regions of interaction with MHC may be different between CD4 and CD8. In contrast to CD4, CD8 is a dimer as is Ig. Interestingly, the two shortened regions in CD4 between strands CC′ and FG are not shortened in CD8. Therefore, the CDR3-like region would be available as a site of interaction in CD8. We found that the mutation in CD8 with the greatest effect on binding to MHC class I, mutant M2, which was a leucine to glycine substitution, was located in the CDR1-like region. Therefore, in our work both the CDR1 and CDR2 regions of CD8 appear to be important for MHC class I binding.

We would therefore postulate that the CDR1- and CDR2-like regions of CD8α are important for species-specific interaction of CD8α with HLA class I and that an additional region such as the CDR3-like region is also involved in binding. Because the CDR3-like region is highly conserved between murine and human and lacks nonconservative changes, this would suggest that it is not involved in species specificity of binding. Instead, this may be the region binding to the conserved loop from position 223–229 in the α3 domain of MHC class I important for CD8 binding (19). The residues in this loop are identical between mouse and human except at position 228, which contains a methionine instead of a threonine. If this region binds to a conserved region of CD8, then the CDR3 domain (Fig. 3, L3) is the region most likely to make contact with the 223–229 loop given the likelihood that CD8 has an Ig-like structure as does CD4, and given the importance of the loops as sites of interaction with other molecules. In addition, our data would support the findings that there are additional amino acids in the α3 domain of HLA important for contact with CD8 besides those in the conserved loop from position 223–229 (19).

From the analyses of T cell responses to MHC class I, a species-specific interaction between murine CD8 and the α3 domain of MHC class I was proposed (43, 44). The majority of A2-specific murine CTL clones induced by stimulation with A2-expressing human stimulator cells showed enhanced recognition of target cells expressing the human-murine chimeric molecule $A2/K^b$, and this enhanced recognition of $A2/K^b$ was sensitive to inhibition by anti-CD8 antibody (43). This was also the case for stimulation of CTL precursors, which implied that properties of the α3 domain were also important for T cell activation. Kalinke et al. (44) produced transgenic mice with chimeric α1 and α2 domains of HLA-B27 and the α3 domain of the mouse Lyt-2 molecule, and demonstrated that the spleen T cells responded to the chimeric molecule at a frequency characteristic of an allogeneic response. They demonstrated that the poor response to

xenogeneic MHC class I molecules resulted from the inability of the mouse CD8 co-receptor to interact with the human MHC molecule. Our results using a cell-cell adhesion assay are consistent with these findings.

In contrast, human CD8α was reported to bind to either murine or human MHC class I molecules (45) using a cell-cell adhesion assay. Murine and human MHC class I molecules were transfected into the human A^-, B^- class I cell line, CIR, and binding to CHO expressing high levels of CD8 was determined. Good binding to either murine or human molecules was observed. However, the conformation of mouse class I heavy chains associated with human β_2-microglobulin may not be equivalent to mouse class I heavy chains paired with mouse β_2-microglobulin. For instance, the human class I W6/32 antibody epitope is lost when β_2-microglobulin from mouse pairs with the human class I heavy chain (46). Further work is required to affirm this difference between the human and mouse CD8 molecule.

This is the first report of a cell-cell adhesion assay using a transient CD8 expression system in COS 7 cells to detect binding to HLA class I–expressing B cells. The lipofectin reagent used in these studies in combination with the pCDL-SRα296 vector gives high level CD8α expression on COS 7 cells and is sufficient to analyze mutants of CD8 and their binding to HLA class I–bearing B cells. We did not find as good CD8α expression in this system using the CDM8 vector (47) (data not shown). In contrast to the CHO cell-cell adhesion assay (17), which takes 2–3 mo to establish high level CD8 expressors, the transient assay is rapid. The transient COS 7 cell-cell adhesion assay gives similar results to the CHO cell-cell adhesion assay. The fold difference between $CD8^+$ and $CD8^-$ transfectants in both systems is similar (four- to ninefold), even though the percent total cells bound is less in the transient assay, because all of the COS 7 cells are not expressing high levels of CD8. Thus, with a transient assay, we can screen mutants rapidly. With the new information on the CD4 crystal structure, models of CD8 based on Ig are more likely to approximate the actual structure. We can now use this information in conjunction with our present findings to design future mutations to more precisely determine sites of interaction with MHC class I.

We thank the following for antibodies: Dr. Herman Waldman (Campath B and 8B), Becton Dickinson & Co.) (Leu-2a, b, c, and d), and Dr. Jeff Ledbetter (51.1, 66.2, G17.5, G19.5, and 53.6). We also thank John Mennone for technical assistance, and Drs. Linda Clayton and Dan Leahy for helpful discussion.

This work was supported by a National Institutes of Health grant (CA-481115). P. Kavathas is a Leukemia Society Scholar.

Address correspondence to Paula Kavathas, Department of Laboratory Medicine, Yale University, 333 Cedar Street, P.O. Box 3333, New Haven, CT 06510.

Received for publication 8 January 1991 and in revised form 7 May 1991.

References

1. Rudd, C.E., J.M. Trevillyan, J.D. Dasgupta, L.L. Wong, and S.F. Schlossman. 1988. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp^{58}) from human T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 85:5190.
2. Barber, E.K., J.D. Dasgupta, S.F. Schlossman, J.M. Trevillyan, and C.E. Rudd. 1989. The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase ($p56^{lck}$) that phosphorylates the CD3 complex. *Proc. Natl. Acad. Sci. USA.* 86:3277.
3. Veillette, A., M.A. Bookman, E.M. Horak, and J.B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{kk}$. *Cell.* 55:301.
4. Emmrich, F., U. Strittmatter, and K. Eichmann. 1986. Synergism in the activation of human CD8 T cells by cross-linking the T-cell receptor complex with the CD8 differentiation antigen. *Proc. Natl. Acad. Sci. USA.* 83:8298.
5. Ledbetter, J.A., C.H. June, L.S. Grosmaire, and P.S. Rabinovitch. 1987. Crosslinking of surface antigens causes mobilization of intracellular ionized calcium in T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 84:1384.
6. Nakayama, T., A. Singer, E.D. Hsi, and L.E. Samelson. 1989. Intrathymic signaling in immature $CD4^+CD8^+$ thymocytes results in tyrosine phosphorylation of the T-cell receptor zeta chain. *Nature (Lond.).* 341:651.
7. Zuniga-Pflucker, J.C., L.A. Jones, D.L. Longo, and A.M. Kruisbeek. 1990. CD8 is required during positive selection of $CD4^-/CD8^+$ T cells. *J. Exp. Med.* 171:427.
8. Brombacher, F., M.C. Lamers, G. Kohler, and H. Eibel. 1989. Elimination of $CD8^+$ thymocytes in transgenic mice expressing an anti-Lyt2.2 immunoglobulin heavy chain gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3719.
9. Sha, W.C., C.A. Nelson, R.D. Newberry, D.M. Kranz, J.H. Russell, and D.Y. Loh. 1988. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature (Lond.).* 336:73.
10. Teh, H.S., P. Kisielow, B. Scott, H. Kishi, Y. Uematsu, H. Bluthmann, and H. von Boehmer. 1988. Thymic major histocompatibility complex antigens and the $\alpha\beta$ T-cell receptor determine the CD4/CD8 phenotype of T cells. *Nature (Lond.).* 335:229.
11. Terry, L.A., J.P. DiSanto, T.N. Small, and N. Flomenberg. 1990. Differential expression and regulation of the human

CD8α and CD8β chains. *Tissue Antigens.* 35:82.
12. Blue, M.L., J.F. Daley, H. Levine, K.A. Craig, and S.F. Schlossman. 1986. Biosynthesis and surface expression of T8 by peripheral blood T4$^+$ cells in vitro. *J. Immunol.* 137:1202.
13. Paliard, X., R.D.W. Malefijt, J.E. de Vries, and H. Spits. 1988. Interleukin-4 mediates CD8 induction on human CD4$^+$ T-cell clones. *Nature (Lond.).* 335:642.
14. Norment, A.M., R.D. Salter, P. Parham, V.H. Engelhard, and D.R. Littman. 1988. Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature (Lond.).* 336:79.
15. Doyle, C., and J.L. Strominger. 1987. Interaction between CD4 and class II MHC molecules mediated cell adhesion. *Nature (Lond.).* 330:256.
16. Clayton, L.K., M. Sieh, D.A. Pious, and E.L. Reinherz. 1989. Identification of human CD4 residues affecting class II MHC versus HIV-gp120 binding. *Nature (Lond.).* 339:548.
17. Rosenstein, Y., S. Ratnofsky, S.J. Burakoff, and S.H. Herrmann. 1989. Direct evidence for binding of CD8 to HLA class I antigens. *J. Exp. Med.* 169:149.
18. O'Rourke, A.M., J. Rogers, and M.F. Mescher. 1990. Activated CD8 binding to class I protein mediated by the T-cell receptor results in signalling. *Nature (Lond.).* 346:187.
19. Salter, R.D., R.J. Benjamin, P.K. Wesley, S.E. Buxton, T.P.J. Garrett, C. Clayberger, A.M. Krensky, A.M. Norment, D.R. Littman, and P. Parham. 1990. A binding site for the T-cell co-receptor CD8 on the α_3 domain of HLA-A2. *Nature (Lond.).* 345:41.
20. Connolly, J.M., T.A. Potter, E.M. Wormstall, and T.H. Hansen. 1988. The Lyt-2 molecule recognizes residues in the class I α3 domain in allogeneic cytotoxic T cell responses. *J. Exp. Med.* 168:325.
21. Potter, T.A., T.V. Rajan, R.F. Dick II, and J.A. Bluestone. 1989. Substitution at residue 227 of H-2 class I molecules abrogates recognition by CD8-dependent, but not CD8-independent, cytotoxic T lymphocytes. *Nature (Lond.).* 337:73.
22. Kabat, E.A., T.T. Wu, M. Reid-Miller, H. Perry, and K.S. Gottesman. 1987. Sequences of Proteins of Immunological Interest. 4th ed. U.S. Department of Health and Human Services, Wash. DC.
23. Margolskee, R.F., P. Kavathas, and P. Berg. 1988. Epstein-Barr virus shuttle vector for stable episomal replication of cDNA expression libraries in human cells. *Mol. Cell. Biol.* 8:2837.
24. Tagawa, M., H. Nakauchi, L.A. Herzenberg, and G.P. Nolan. 1986. Formal proof that different-size Lyt-2 polypeptides arise from differential splicing and post-transcriptional regulation. *Proc. Natl. Acad. Sci. USA.* 83:3422.
25. Takebe, Y., M. Seiki, J. Fujisawa, P. Hoy, K. Yokota, K. Arai, M. Yoshida, and N. Arai. 1988. SRα Promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* 8:466.
26. Kunkel, T.A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA.* 82:488.
27. Yokota, T., F. Lee, D. Rennick, C. Hall, N. Arai, T. Mosmann, G. Nabel, H. Cantor, and K. Arai. 1984. Isolation and characterization of a full-length cDNA for mast cell growth factor from a mouse T cell clone: expression in monkey cells. *Proc. Natl. Acad. Sci. USA.* 81:1070.
28. Felgner, P.L., T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, and M. Danielson. 1987. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA.* 84:7413.
29. Glassy, M.C., H.H. Handley, H. Hagiwara, and I. Royston. 1983. UC 729-6, a human lymphoblastoid B-cell line useful for generating antibody-secreting human-human hybridomas. *Proc. Natl. Acad. Sci. USA.* 80:6327.
30. Reinherz, E.L., and S.F. Schlossman. 1980. The differentiation and function of human T lymphocytes. *Cell.* 19:821.
31. Ledbetter, J.A., and L.A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* 47:63.
32. Kavathas, P., and L.A. Herzenberg. 1983. Stable transformation of mouse L cells for human membrane T-cell differentiation antigens, HLA and β_2-microglobulin: selection by fluorescence-activated cell sorting. *Proc. Natl. Acad. Sci. USA.* 80:524.
33. Staunton, D.E., M.L. Dustin, H.P. Erickson, and T.A. Springer. 1990. The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. *Cell.* 61:243.
34. Williams, A.F., and A.N. Barclay. 1988. The immunoglobulin superfamily: domains for cell surface recognition. *Annu. Rev. Immunol.* 6:381.
35. Kirszbaum, L., J.A. Sharpe, N. Goss, J. Lahnstein, and I.D. Walker. 1989. The α-chain of murine CD8 lacks an invariant Ig-like disulfide bond but contains a unique intrachain loop instead. *J. Immunol.* 142:3931.
36. Chothia, C., and A.M. Lest. 1987. Canonical Structures for the Hypervariable Regions of Immunoglobulins. *J. Mol. Biol.* 196:901.
37. Epp, O., E.E. Lattman, M. Schiffer, R. Huber, and W. Palm. 1975. The molecular structure of a dimer composed of the variable portions of the Bence-Jones protein REI refined at 2.0-A resolution. *Biochemistry.* 14:4943.
38. Ponder, J.W., and F.M. Richards. 1987. Tertiary templates for proteins use of packing criteria in the enumeration of allowed sequences for different structural proteins. *J. Mol. Biol.* 193:775.
39. Lamarre, D., A. Ashkenzai, S. Fleury, D.H. Smith, R.P. Sekaly, and D.J. Capon. 1989. The MHC-binding and gp120-binding functions of CD4 are separable. *Science (Wash. DC).* 245:743.
40. Peterson, A., and B. Seed. 1988. Genetic analysis of monoclonal antibody and HIV binding sites on the human lymphocyte antigen CD4. *Cell.* 54:65.
41. Wang, J., Y. Yan, T.P.J. Garrett, J. Liu, D.W. Rodgers, R.L. Garlick, G.E. Tarr, Y. Husain, E.L. Reinherz, and S.C. Harrison. 1990. Atomic structure of a fragment of human CD4 containing two immunoglobulin-like domains. *Nature (Lond.).* 348:411.
42. Ryu, S.E., P.D. Kwong, A. Truneh, T.G. Porter, J. Arthos, M. Rosenberg, X. Dai, N. Xuong, R. Axel, R.W. Sweet, and W.A. Hendrickson. 1990. Crystal structure of an HIV-binding recombinant fragment of human CD4. *Nature (Lond.).* 348:419.
43. Irwin, M.J., W.R. Heath, and L.A. Sherman. 1989. Species-restricted interactions between CD8 and the α3 domain of class I influence the magnitude of the xenogeneic response. *J. Exp. Med.* 170:1090.
44. Kalinke, U., B. Arnold, and G. Hammerling. 1990. Strong xenogeneic HLA response in transgenic mice after introducing an α3 domain into HLA B27. *Nature (Lond.).* 642:642.
45. Salter, R.D., A.M. Norment, B.P. Chen, C. Clayberger, A.M. Krensky, D.R. Littman, and P. Parham. 1989. Polymorphism in the α3 domain of HLA-A molecules affects binding to CD8. *Nature (Lond.).* 338:345.
46. Ferrier, P., J.C. Fontecilla-Camps, D. Bucchini, D.H. Caillol,

B.R. Jordan, and F.A. Lemonnier. 1985. Altered structure of HLA class I heavy chains associated with mouse beta-2 microglobulin. *Immunogenetics.* 21:321.

47. Aruffo, A., and B. Seed. 1987. Molecular cloning of a CD28 cDNA by a high efficiency COS cell expression system. *Proc. Natl. Acad. Sci. USA.* 84:8573.
48. Nakauchi, H., G.P. Nolan, C. Hsu, H.S. Huang, P. Kavathas, and L.A. Herzenberg. 1985. Molecular cloning of Lyt-2, a membrane glycoprotein marking a subset of mouse T lymphocytes: molecular homology to its human counterpart, Leu-2/T8 and to immunoglobulin variable regions. *Proc. Natl. Acad. Sci. USA.* 82:5126.
49. Priestle, J.P. 1988. RIBBON: a stereo cartoon drawing program for proteins. *J. Appl. Cryst.* 21:572.