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The role of charge and multiple faces of the CD8α/α homodimer in binding to major histocompatibility complex class I molecules: Support for a bivalent model

PATRICIA A. GIBLIN*, DANIEL J. LEAHY†, JOHN MENNONE*, AND PAULA B. KAVATHAS*‡

*Section of Immunobiology and Department of Laboratory Medicine, Yale University, New Haven, CT 06520-8035; †Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032; and ‡Department of Genetics, Yale University, New Haven, CT 06520-8035

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ABSTRACT **The CD8 dimer interacts with the α3 domain of major histocompatibility complex class I molecules through two immunoglobulin variable-like domains. In this study a crystal structure-informed mutational analysis has been performed to identify amino acids in the CD8α/α homodimer that are likely to be involved in binding to class I. Several key residues are situated on the top face of the dimer within loops analogous to the complementarity-determining regions (CDRs) of immunoglobulin. In addition, other important amino acids are located in the A and B β-strands on the sides of the dimer. The potential involvement of amino acids on both the top and the side faces of the molecule is consistent with a bivalent model for the interaction between a single CD8α/α homodimer and two class I molecules and may have important implications for signal transduction in class I-expressing cells. This study also demonstrates a role for the positive surface potential of CD8 in class I binding and complements previous work demonstrating the importance of a negatively charged loop on the α3 domain of class I for CD8α/α–class I interaction. We propose a model whereby residues located on the CDR-like loops of the CD8 homodimer interact with the α3 domain of MHC class I while amino acids on the side of the molecule containing the A and B β-strands contact the α2 domain of class I.**

The cell surface glycoprotein CD8 is involved in mediating the adhesion of mature CD8$^+$ T lymphocytes to their major histocompatibility complex (MHC) class I-expressing targets. CD8 acts as a coreceptor by binding to the α3 domain of MHC class I protein and stabilizing the interaction of the T-cell receptor with antigenic peptide presented in the α1/α2 cleft of the same class I molecule (1–3). Once bound, CD8 contributes to the transduction of intracellular signals that are important for activation (4–6). Effective CD8–class I interactions are also required for the maturation of T cells within the thymus, as evidenced by *in vivo* antibody inhibition studies as well as studies with transgenic animals lacking CD8 or expressing class I molecules incapable of efficiently engaging CD8 (7–11).

Recently the crystal structure of a soluble form of the human CD8α/α homodimer was solved (12). In the N-terminal domain of each polypeptide, two sheets consisting of five and four antiparallel β-strands form a typical immunoglobulin fold. Connecting these strands is a conserved array of loops, three of which are analogous to the complementarity-determining regions of immunoglobulin (CDR1, CDR2, CDR3). Modeling of the electrostatic surface potential of the CD8α/α homodimer has revealed that the membrane-distal, CDR-containing surface of the molecule is predominantly positively charged.

The binding site on class I molecules for CD8α/α has been studied in detail. Previous mutational analyses have identified three clusters of amino acids in the α3 domain that are important for CD8α/α–class I binding and pinpointed one cluster, residues 223–229, as critical for this interaction (1, 13–15). This cluster is conserved between mouse and human and comprises a highly exposed, negatively charged loop between strands C and D in the α3 domain. In contrast to the body of work that has contributed to the localization of the putative class I binding site for CD8, only one study has focused on defining the complementary interaction site on CD8α/α. Based on the finding that murine CD8α/α did not bind to human HLA class I in a cell–cell adhesion assay, Sanders *et al.* (16) performed homolog scanning mutagenesis by exchanging human amino acids with their nonconserved murine counterparts. The results of this work indicated a role for both the CDR1-like and CDR2-like loops in this species-specific interaction. However, since this study focused on the differences between two species, conserved amino acids involved in binding would not have been identified. In addition, the individual amino acids which might be potential contact points could not be determined because these mutants often contained more than one amino acid substitution.

In the current study the site of interaction on CD8α/α has been further characterized by using a panel of point mutants generated with the aid of the CD8α/α crystal structure. Analysis of these mutants in a transient cell–cell adhesion assay has identified a core of amino acids that are likely candidates for interaction with class I molecules. Overall, the results support a bivalent model of interaction between one CD8 homodimer and two class I molecules. The role of electrostatic interactions between the predominantly positively charged CD8α/α and the predominantly negatively charged class I α3 domain is also supported by these findings.

MATERIALS AND METHODS

Mutagenesis. Site-directed mutagenesis of the human CD8α cDNA in pBluescript SK(+) was as described (16). To facilitate the sequencing and subcloning of the mutants, *Sac* I and *Sac* II restriction sites (silent mutations) were introduced to flank the immunoglobulin variable-like domain near Leu^{20} (L20) and P122, respectively. The majority of mutants were sequenced in pBluescript between the *Sac* I and *Sac* II restriction sites, excised with *Sac* I and *Sac* II, and subcloned into *Sac* I/*Sac* II-digested expression vector pCDL-SRα296 (17) containing the *Sac* I/*Sac* II-modified CD8α cDNA. The only exceptions were mutants R4A and R4E, which were sequenced and subcloned between the *Pst* I (5′ untranslated) and *Sac* I sites, excised from the same sites.

Expression of Mutant Forms of CD8α/α in COS-7 Cells. COS-7 cells were maintained as described (16) and transfected with wild-type or mutant forms of CD8α cDNA by a modified lipofection protocol. For each plate, 5 μg of plasmid

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Abbreviations: CDR, complementarity-determining region; MHC, major histocompatibility complex.

and 20 μl of Lipofectin (GIBCO/BRL) were separately diluted to 100 μl in Opti-MEM and then mixed. After 20 min at room temperature, the lipofection mix was diluted by adding 0.8 ml of Opti-MEM containing 1.25% fetal bovine serum and added to a 35-mm dish of nearly confluent COS-7 cells. Lipofection was stopped after 18 hr by replacing the lipofection mix with 2 ml of fresh medium containing 10% fetal bovine serum. Lipofectants were fed again after 24 hr, and after another 24 hr the dishes were either analyzed for cell surface expression of CD8α or used in the adhesion assay. Staining was as described (16).

Cell–Cell Adhesion Assay. Binding assays were as described (16) with the exception that UC cells stably expressing the luciferase gene were used. This UC-LUC cell line was prepared by transfecting the UC cells with the Epstein–Barr virus-based plasmid p220LUC expressing the firefly luciferase gene under the control of 618 bp of the human CD8α promoter (19) and selecting with hygromycin B at 300 μg/ml. The HLA antigens expressed by the UC cells were A1, A2, B5[51], B57, Cw4, DR7, DQ2, and DQ3. Bound cells were lysed at room temperature for 10 min in 300 μl of 1× lysis buffer (Duc-luciferin luciferase kit, Promega). Lysates were collected and the plates were washed with 300 μl of lysis buffer. Each sample was centrifuged and 20 μl of supernatant was assayed for luciferase activity with 100 μl of Duc-luciferin assay buffer in a luminometer (Berthold, Nashua, NH). To generate a standard curve, lysates were prepared from known numbers of UC-LUC cells mixed with COS-7 cells from confluent 35-mm dishes. For each experiment, the binding assay was performed two to three times per mutant or three to four times for vector alone or vector containing wild-type CD8α. Each mutant was analyzed in two to five experiments. Mean values for replicate plates within one experiment were calculated and corrected for UC-LUC binding to COS-7 cells transfected with vector alone and binding was expressed as the percent wild-type binding. Results are presented as a composite of the results for several experiments.

RESULTS

To further define the molecular interactions between MHC class I and the CD8α/α homodimer, a detailed mutational analysis of CD8α was undertaken using the available crystal structure. We examined three major issues concerning CD8α–class I interaction: (*i*) the potential existence of species-conserved residues that participate in adhesion, (*ii*) the potential involvement of faces other than the CDR-like face of the homodimer in binding, and (*iii*) the role of electrostatic interactions in binding. For mutagenesis we chose amino acids whose side chains were highly solvent accessible, reasoning that these residues were most likely to make contact with MHC class I molecules and that changes at these positions were least likely to result in global structural alterations. To better interpret the results from our mutational analysis, we made two amino acid substitutions at each of the chosen positions whenever possible. The first choice for substitution was alanine and the second an amino acid whose side chain differed in size and/or charge from the original. Fig. 1 is a ribbon diagram of the CD8α/α homodimer with the amino acids chosen for mutation labeled on one or the other CD8α immunoglobulin-variable-like domain.

For analysis of these mutants, a transient cell–cell adhesion assay was employed. In this system wild-type and mutant forms of CD8α are expressed in simian virus 40-transformed COS-7 monkey cells (21) and then tested for their ability to bind to the class I^+ B-lymphoblastoid cell line UC (23). Since the levels of cell surface expression varied from experiment to experiment with this assay, expression of each mutant was determined for a replicate plate in each experiment and

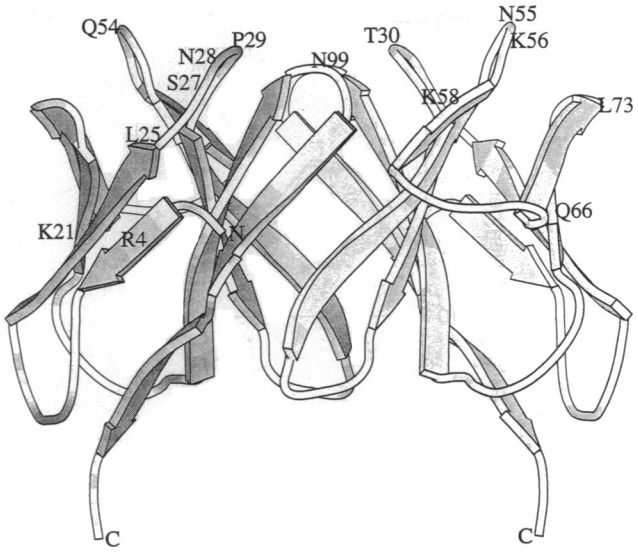

FIG. 1. Location of mutations made in human CD8α. Shown is a ribbon diagram of the CD8α/α homodimer with the positions of mutations labeled (22). β-Strands are arranged as in immunoglobulin variable domains (amino terminus, A, B, C, C′, C″, D, E, F, G, carboxyl terminus). The CDR-like loops occupy the top surface of the molecule, and the dyad axis of symmetry is parallel to the plane of the page and passes between the CDR3-like (N99) and C–C′ loops (bottom). Residues 27–30 are situated in the CDR1-like loops, and residues 54–56 and 58 are located on opposite sides of the CDR2-like loops. The "front" face of the left monomer contains R4, of strand A, and K21 and L25, both of which are in strand B. The complementary residues of the opposing monomer would be present on the "back" face of the right domain but are not visible in this orientation of the molecule. Conversely, Q66 and L73 are located on the front face of the right monomer with corresponding residues on the back surface of the left monomer.

compared with wild-type CD8α expression on that day. Mutant expression levels were 75–150% of wild-type CD8α as judged by mean fluorescence intensity, and all mutants were expressed similar to wild type in at least one experiment. Typical expression patterns are shown in Fig. 2. To ascertain that the introduced mutations did not grossly alter the structure of the CD8α/α homodimer, each mutant was stained with a panel of four or five anti-CD8α antibodies. Very few of the mutants showed noticeable loss of an antibody epitope, although mutants K21E, L73A, and L73E, whose side chains are proximally located to one another, were unable to bind anti-Leu-2a (data not shown). A subset of the mutants were also analyzed by immunoprecipitation to determine whether they were properly expressed as homodimers and to determine the extent to which they formed high molecular weight complexes on COS-7 cells. This subset included residues from the CDR-containing face as well as the A/B and the C″/D sides of the molecule. Of those mutants analyzed, all were expressed as homodimers, and higher molecular weight complexes were not observed. Mutant L25N, which gained an N-linked glycosylation site, exhibited a corresponding increase in the molecular weight of a proportion of the labeled L25N mutant molecules (data not shown).

These binding experiments showed that several amino acids located on the top and on the A/B strand side of the molecule were important for binding (Fig. 3). Of particular interest were four amino acids—R4, L25, K58, and N99—whose replacement by either alanine or an amino acid varying in charge or size resulted in complete inhibition of binding. R4 and L25 are located on the side of the molecule on the A and the B strands, respectively. In the crystal structure the positively charged side chain of R4 is highly exposed and

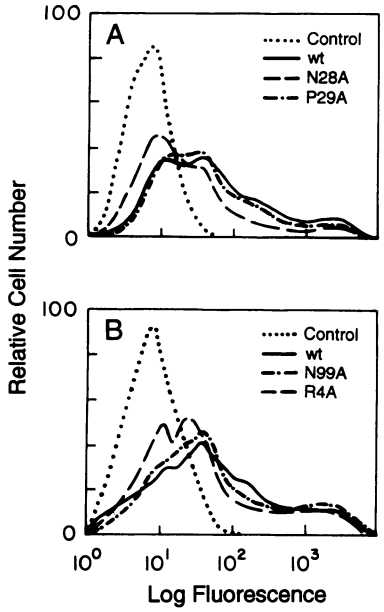

FIG. 2. Transient expression of wild-type CD8α and mutant forms of CD8α in COS-7 cells. Shown are representative flow cytofluorimetry profiles from two different experiments in which samples were stained with the anti-CD8 antibodies OKT8 (*A*) or Leu-2a (*B*) plus fluoresceinated goat anti-mouse IgG (16). Mutants P29A and N99A were expressed similar to wild type (wt) in these experiments while mutants R4A and N28A were expressed at slightly lower levels. Despite this decreased level of expression of N28A, this mutant still exhibited binding equivalent to 62% of that seen with wild-type CD8α.

immediately adjacent to the side chain of the hydrophobic L25 residue. In contrast, K58 and N99 are situated on the top surface of the homodimer in the CDR2 and CDR3 loops, respectively, and K58 from one monomer is close to N99 from the other (Fig. 1). L25, K58, and N99 are conserved among human, bovine, rat, and mouse CD8α and may, therefore, constitute part of a core of conserved amino acids involved in the binding of CD8α to MHC class I.

The importance of charge in CD8α/α–class I interactions was also demonstrated. Two of the four critical amino acids, R4 and K58, are positively charged. The placement of a negative charge at S27 and N28 within the CDR1-like loop and at Q54 and N55 in the CDR2-like loop also led to complete inhibition of binding (Fig. 3*B*), whereas substitution with alanine at these same positions had minimal effects (Fig. 3*A*). Replacement of the positively charged K21 residue (B strand) with glutamic acid also led to complete inhibition of binding, while substitution of glutamic acid for K56 led to intermediate levels of binding, suggesting that K56 does not make direct contact with class I and that the positively charged side chain of K56 is not critical for effective electrostatic interactions between CD8α/α and class I. In striking contrast, the Q66E mutant (C″ to D loop) and the L73A or L73E mutant (D to E loop), which are located on a different face, had minimal effects on binding. Other previous mutations near L73 in the D to E loop also had minimal effects on binding (16).

Further support for the potential involvement of the CDR1-like loop in binding is provided by our analysis of mutant P29A, which also failed to bind to class I. This amino acid may make direct contact with class I or the observed effect may be caused by a more global conformational change in the CDR1-like loop. Since the proline ring may constrain the conformation of the CDR1-like loop, the substitution of alanine for proline at this position may result in added flexibility within the loop, thus allowing the loop to adopt different conformations.

Based on these findings, we have generated a topographic representation to depict the regions of the CD8α/α homodimer that are important for binding to class I. The proposed surface of contact spans the top face of the homodimer and also includes several residues on the A and B strands (Fig. 4 *A* and *B*). The involvement of this large surface area and opposite sides of the CD8α/α homodimer is not inconsistent with a bivalent model for the binding of CD8 to class I (12). If there are, in fact, two sites of interaction, then we propose that R4 (A strand), L25 (B strand), and N99 (CDR3-like loop) from one monomer and K58 (CDR2-like loop) from the opposing monomer constitute part of a core of amino acids involved in binding a single class I molecule.

We generated a model to illustrate how one CD8 homodimer may interact with two MHC class I molecules. Using the available mutational data form both CD8α/α and MHC class I, we assumed that (*i*) the positively charged CDR-like loops of CD8 would interact with the negatively charged 223–229 loop in the α3 domain of class I and (*ii*) the CDR-like loops of CD8 which could be furthest away from the cell membrane would contact the membrane-proximal α3

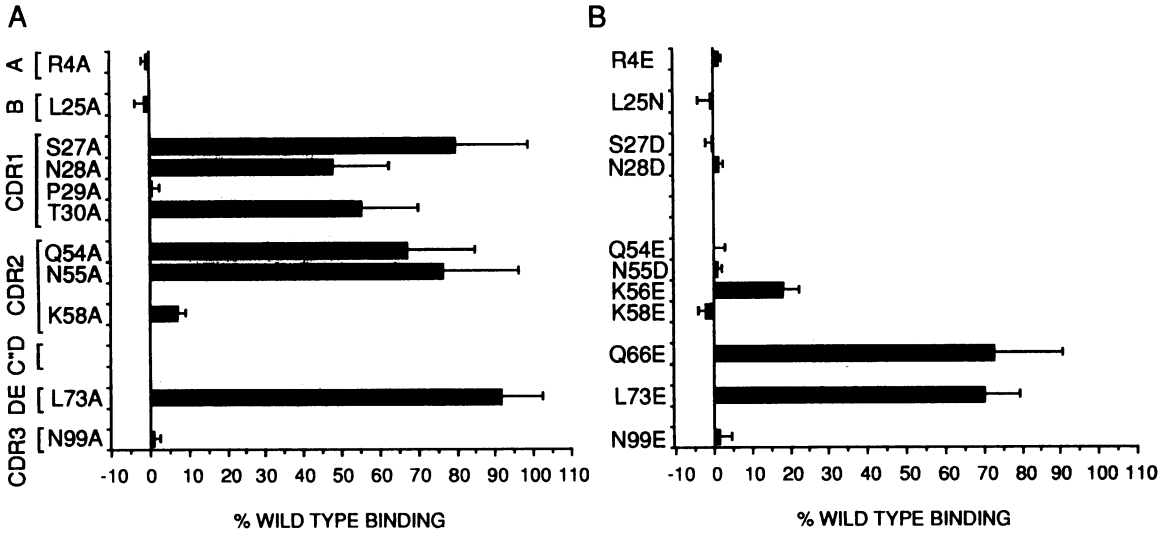

FIG. 3. Binding of mutant forms of CD8α to HLA class I relative to wild-type binding. Alanine substitution (*A*) and size/charge substitution (*B*) mutants are shown. Error bars represent SEM.

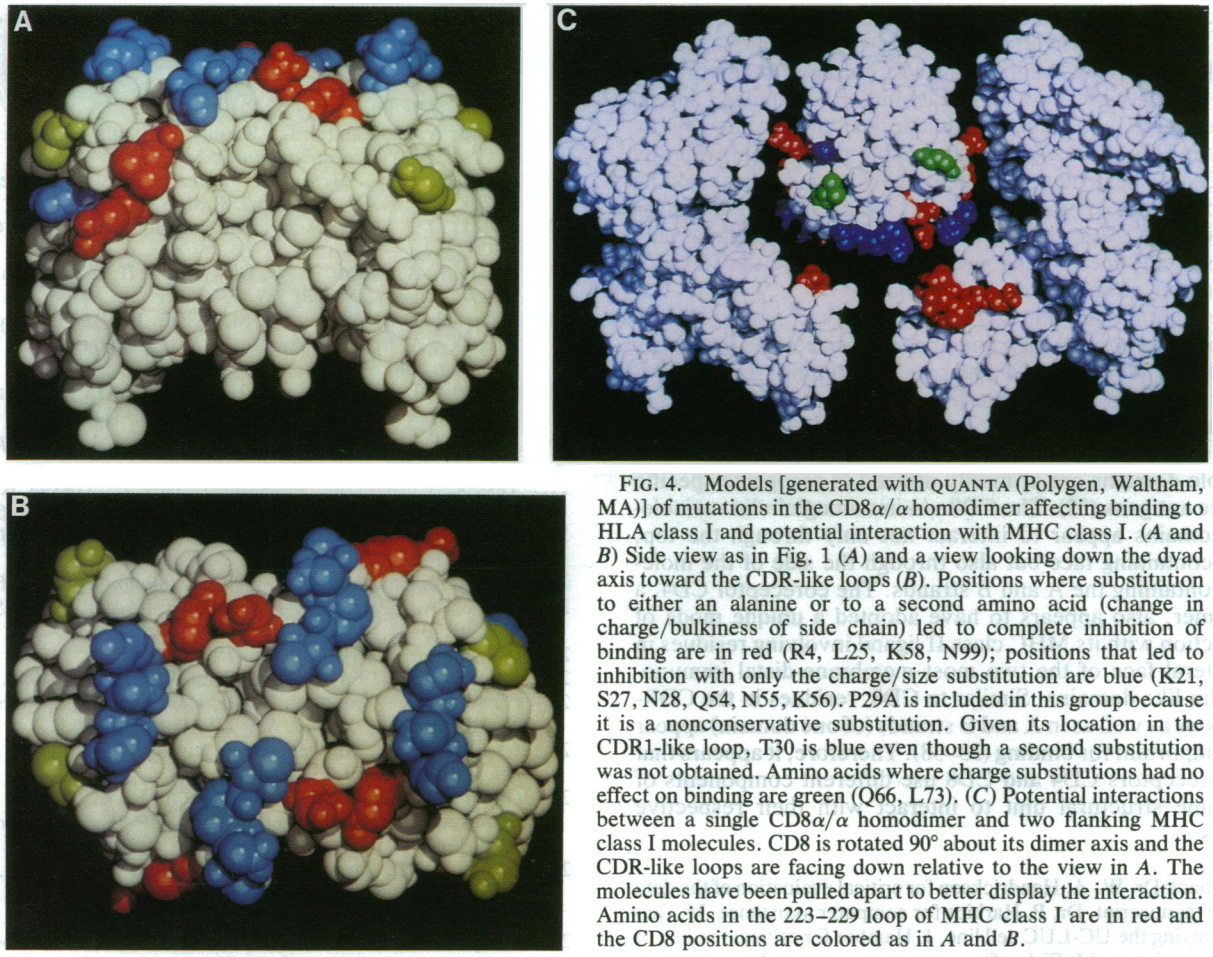

FIG. 4. Models [generated with QUANTA (Polygen, Waltham, MA)] of mutations in the CD8α/α homodimer affecting binding to HLA class I and potential interaction with MHC class I. (*A* and *B*) Side view as in Fig. 1 (*A*) and a view looking down the dyad axis toward the CDR-like loops (*B*). Positions where substitution to either an alanine or to a second amino acid (change in charge/bulkiness of side chain) led to complete inhibition of binding are in red (R4, L25, K58, N99); positions that led to inhibition with only the charge/size substitution are blue (K21, S27, N28, Q54, N55, K56). P29A is included in this group because it is a nonconservative substitution. Given its location in the CDR1-like loop, T30 is blue even though a second substitution was not obtained. Amino acids where charge substitutions had no effect on binding are green (Q66, L73). (*C*) Potential interactions between a single CD8α/α homodimer and two flanking MHC class I molecules. CD8 is rotated 90° about its dimer axis and the CDR-like loops are facing down relative to the view in *A*. The molecules have been pulled apart to better display the interaction. Amino acids in the 223–229 loop of MHC class I are in red and the CD8 positions are colored as in *A* and *B*.

domain of class I. Given this interaction, the "R4–L25" side of CD8 would bind to HLA class I on the α2 surface above 223–229 loop. When the molecules are arranged in this fashion, contacts consistent with all of the mutation data are made. In addition two molecules of HLA class I can interact symmetrically with one CD8 dimer in an orientation analogous to the orientation found in crystal structures for dimers of HLA class II molecules.

DISCUSSION

From the available CD8α/α crystal structure, mutants have been designed and assayed for their ability to bind to MHC class I molecules in a cell–cell adhesion assay. The results indicate a role for residues on the membrane-distal, CDR-containing face as well as on the A/B-strand side of the dimer. The role of charge interactions is demonstrated, as the removal of key positively charge amino acids (R4, K58) or the addition of an acidic residue to the CDR face severely affects binding.

The results support a model in which a single CD8α/α homodimer exhibits two binding sites for class I. Such a bivalent model has interesting implications for the biology of CD8–MHC class I interaction. Having two binding sites for class I would potentially allow the CD8α/α homodimer to crosslink two human class I molecules on the surface of a target cell. Since crosslinking of MHC class I with antibodies can lead to the transduction of intracellular signals (24, 25), this model suggests a mechanism through which signals may be delivered to the target cell during interaction with a CD8-expressing cell. Additionally, this CD8–class I clustering may contribute to triggering of effector cells. This type of cell surface crosslinking may also be important for the interaction of the coreceptor CD4 with its ligand, MHC class II. The recent crystallization of human class II HLA-DR1 as dimers of the α/β heterodimer has led to the proposal that these dimers exist on class II-expressing cells and facilitate the aggregation of the T-cell receptor–CD4 complex (26).

Another prediction that can be made from the model is that regions of the MHC class I α2 domain and perhaps β_2-microglobulin are involved in CD8 binding. At least one of these residues may be negatively charged to facilitate interaction with the positively charged R4 residue on CD8. In our model it is possible that the α3 domains of two MHC class I molecules may also interact within the complex but that the interaction is too small to result in dimerization in the absence of CD8.

Two molecular forms of human CD8 exist, a homodimer consisting of two identical α chains and a heterodimer composed of one α and one β chain. Both forms are coordinately expressed on the majority of human CD8$^+$ T cells in proportions that vary from individual to individual (27, 28). In contrast, other cells exclusively express the homodimeric form of CD8. These include the majority of α/β and γ/δ T-cell-receptor-positive intraepithelial lymphocytes of the intestinal mucosa and a subset of human natural killer cells (29). The CD8α/α homodimer may also be induced on mature CD4$^+$ T cells following treatment with interleukin 4 or phytohemagglutinin (28, 30). Based on the differential expression of these two forms of human CD8, it has been proposed that the CD8 homo- and heterodimer may be functionally distinct. The amino acids in the immunoglobulin-like domains of CD8α and CD8β are only 17.5% identical, and the class I-interacting residues defined here are not conserved between CD8α and CD8β. It is therefore possible that these differences may impact on the relative affinity and/or

valency of interaction between MHC class I and the two forms of CD8. Intriguingly, human growth hormone, which complexes with two human growth hormone receptor proteins, possesses two nonidentical binding sites (sites I and II) that sequentially engage nearly identical binding sites on the receptor (20). This suggests a possible mechanism whereby the CD8α/β heterodimer containing two nonidentical binding sites may engage nearly identical binding sites on two class I molecules. Alternatively, the CD8α/β heterodimer may possess only one binding site for class I while the CD8α/α homodimer possesses two.

The immunoglobulin gene superfamily contains several members, many of which have been implicated as mediators of cell–cell adhesion (31). The putative binding site proposed here represents a departure from the typical binding site of immunoglobulin molecules in which a combination of the CDR loops on the top face of the heavy- and light-chain variable domains are primarily utilized to recognize specific soluble antigens (32). The CD8α/α immunoglobulin variable-like domains appear to interact not only through the top, CDR-containing face but also through the side of the molecule containing the A and B strands. The coreceptor CD4, a monomer, also appears to have adopted a unique mode of interaction with its MHC class II ligand involving residues in the lateral face of the two most membrane-distal immunoglobulin-like domains. Similar to CD8, residues in the CDR-like loops as well as in A and B strands (of one domain) appear to be important for binding (33–38). Therefore, it appears that the coreceptors CD4 and CD8 use different components of the same structural unit to interact with their respective ligands.

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