Identification of the CD8 DE Loop as a Surface Functional Epitope

IMPLICATIONS FOR MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I BINDING AND CD8 INHIBITOR DESIGN

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We used an approach of protein surface epitope mapping by synthetic peptides to analyze the surface structure-function relationship of the CD8 protein. Small synthetic peptide mimics of the CD8 DE loop were shown to effectively block CD8 binding to major histocompatibility complex (MHC) class I molecules and possess significant inhibitory activity on \textit{in vitro} CD8$^+$ T cell function. These results suggested that the DE loop region of the CD8 protein is an important functional epitope mediating CD8-MHC class I interaction and the activation of CD8$^+$ T cells, a finding that is consistent with the recently reported crystal structure of the CD8-MHC class I complex. The structural basis for the biological activity of the DE loop peptide was further analyzed in a series of analogs containing alanine substitutions. This study provides support for the concept of bioactive peptide design based on protein surface epitopes and suggests that such an approach may be applicable to other protein-protein complexes, particularly those of immunoglobulin superfamily molecules.

Human CD8 (hCD8) is a glycoprotein expressed on the surface of T cells that have specificity for antigens presented by MHC class I proteins, found on most tissue types (8). The hCD8 exists as a disulfide-linked $\alpha\alpha$ homodimer and $\alpha\beta$ heterodimer. Both hCD8$\alpha$- and $\beta$-chains contain an amino-terminal immunoglobulin(Ig)-like extracellular domain and a transmembrane cytoplasmic segment (9–12). MHC class I is a heterodimeric glycoprotein that consists of an $\alpha$-chain, with three extracellular domains (designated $a_1$, $a_2$, and $a_3$) and a transmembrane cytoplasmic domain (13) associated with a $\beta_2$-microglobulin ($\beta_2m$). The hCD8 protein is known to mediate two critical functions: 1) it can act as a co-receptor with the T cell receptor (TCR) for antigen by binding to the MHC class I molecule on the antigen-presenting cell, thereby increasing the avidity of the TCR for its ligand (14–17); and 2) it has signal transduction capabilities, as studies have indicated that the cytoplasmic tail of hCD8 is associated with the Src-like tyrosine kinase p56$^{ck}$ (18).

CD8$^+$ T cells are key components of the protective immune response to infectious pathogens, functioning as cytolytic T lymphocytes (CTL) and as important sources of cytokines, including interferon $\gamma$. However, CD8$^+$ T cells are also involved in deleterious responses related to cellular and organ transplantation reactions. For this reason, small molecular agents that regulate the activity of these CD8$^+$ T cells may have important clinical applications.

In general, it is believed that the surface binding sites of Ig-related molecules involve a combination of three complementarity-determining regions (CDRs) (19). Mutational and synthetic peptide studies of CD8 have demonstrated the involvement of the CDR loops in MHC class I recognition (20–22). In contrast, the roles of other surface regions of the CD8 molecule are less well defined, and it has been suggested that the side of the molecule containing the A and B strands (21) may also be important for MHC class I interaction. A similar observation was made in the CD4 protein, where the CC’ loop located on the side of the molecule formed an integrated binding pocket with other CDR loops located on the top face (6, 23). To test this hypothesis in relation to the potential functional role of CD8 surface regions other than the CDR loops, in the present study we used an approach of protein surface epitope mapping with small conformationally constrained peptides mimicking various protein surface loops as specific probes of the structure-function of the corresponding sites in the native molecule. This strategy had been previously useful in identifying new functional surfaces of the CD4 protein (5, 7).

A theoretical analysis to search for surface protruding regions as potential candidates for hCD8$\alpha$ functional epitopes was initially carried out based on the assumption that these highly exposed sites are most likely to be able to participate in molecular recognition. This was followed by the design, synthe-
sis, and biological assay of peptides that may mimic putative binding sites. The results from these studies suggested that the DE loop, together with other well studied CDR loops, were involved in the binding to MHC class I molecules and the activation of CD8+ T cells. Furthermore, the proposed role of the DE loop in mediating CD8 function was consistent with the recently reported crystal structure of the CD8-MHC class I complex (24). Of most importance, the peptide analogs derived from the CD8 DE loop, as described here, may represent promising leads for the further development of novel immunotherapeutic agents.

**EXPERIMENTAL PROCEDURES**

Peptide Synthesis—The peptides were prepared by solid phase synthesis using Fmoc strategy on a 430A peptide synthesizer (Applied Biosystems, Foster City, CA) and a 9050 Pepsynthesizer Plus (Perseptive Biosystems, Cambridge, MA), as described previously (5, 7). A 4-fold excess of N,N,N',N'-tetramethyluronium hexafluorophosphate, and 1-hydroxybenzotriazole, and a 10-fold excess of diisopropylethylamine were used in every coupling reaction step. Removal of the NH2-terminal Fmoc group was

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**TABLE I**

**Theoretical analysis of surface loop regions of hCD8 protein**

| hCD8 loop regions | Residue numbers | Amino acid sequences | RSAaverage |
|-------------------|-----------------|----------------------|------------|
| CDR1              | 24–32           | VLLSNPTSG            | 0.46       |
| CDR2              | 53–58           | YLSQNKPK             | 0.47       |
| CDR3              | 97–102          | LSNSIM               | 0.31       |
| C'D loop          | 58–68           | KAAEGLDTQRF          | 0.45       |
| DE loop           | 71–78           | KRLGDFTFV            | 0.47       |

The protein structural analysis was based on the crystal structure of hCD8αα (31). The modeling study was carried out by using the molecular modeling package of Insight II software (Biosym Inc., San Diego, CA) on a Silicon Graphics Indigo2 workstation. The β-turns within the sequences are underlined and shown in boldface type. RSAaverage stands for the averaged relative solvent accessibility. It was calculated by using the formula RSAaverage = ΣRSASi/n, where RSASi is the relative solvent accessible surface area of the ith residue within the given sequence as compared with its value in a tripeptide Ala-Xaa-Ala sequence (28). n is the number of residues within the sequence.
Identification of CD8 DE Loop as Surface Functional Epitope

accomplished by 20% piperidine in N,N-dimethylformamide. The cleavage of peptides from the resin was carried out with reagent K (25) for 2 h at room temperature with gentle stirring. Crude peptides were then precipitated in ice-cold methyl t-butyl ether, centrifuged, and lyophilized. The cyclization of disulfide cyclic peptides was achieved by using a modified procedure of Misicka et al. (26). Crude peptides were then purified by preparative reverse phase high performance liquid chromatography using a Dynamax 300Å C18 25 cm x 21.4 mm, inner diameter column with a flow rate of 9 ml/min and two solvents systems of 0.1% trifluoroacetic acid/H2O and 0.1% trifluoroacetic acid/acetonitrile. Fractions containing the appropriate peptide were pooled together and lyophilized. The purity of the final products was assessed by analytical reverse phase high performance liquid chromatography, capillary electrophoresis, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Cell Adhesion Assay—The inhibition of hCD8-MHC class I binding by the peptide analogs derived from the hCD8 protein was assessed in a standard cell adhesion assay that reflects this functional interaction (21). Following a modified procedure of Moebius et al. (27), 5 x 10^4 COS-7 cells/well of a 6-well plate were transfected with the T8-pcDNA plasmid, which was constructed by subcloning 1.5 kilobase pairs of cDNA encoding the full-length transmembrane hCD8 in T8-pMV7 (R. Axel, the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) into the EcoRI site of the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA), by DOSPER liposomal transfection reagent (Boehringer Mannheim) according to the supplier’s protocol. Usually, at 48-h post-transfection, 30–40% of the COS-7 cells expressed hCD8, as defined by immunofluorescence. MHC class I-expressing Raji cells (10^7 in 1 ml of RPMI medium with 10% fetal calf serum and 200 μM glutamine) were then added to each well in the presence of peptide at 37 °C for 1 h, followed by extensive washing (6 x). Rosette formation between Raji cells and transfected COS-7 cells was scored microscopically at 100-fold magnification by counting the number of rosettes containing more than 5 Raji cells in 10 random optical fields for each individual well. The positive control without peptide exhibited 300–400 rosettes/well, and the percent inhibition of rosette formation for each experimental peptide was determined by the ratio of the number of rosettes obtained in the presence of the peptide to the number of rosettes in the positive control. COS-7 cells transfected with pcDNA3 vector alone served as a negative control for rosette formation.

Human Cell-mediated Lympholysis (CML) Assay—The peptides were tested for their effect on CD8+ T cell function in a human in vitro CML assay. Allogenic-specific human CTL were generated by co-culturing purified human peripheral blood lymphocyte responder cells (2 x 10^6; purified from donor blood by standard procedures) with irradiated (30 Gy) peripheral blood lymphocytes from a second donor (4 x 10^6) in a 2 ml volume of RPMI medium containing 10% human serum, l-glutamine and penicillin/streptomycin. After 6 days of incubation at 37 °C, 7% CO2, CTL activity was measured in a standard 4-h 51Cr-release CML assay using 51Cr-labeled peripheral blood lymphocyte (second donor) targets at effector:target (E:T) cell ratios of 50:1 and 25:1. Percent-specific lysis was determined as the mean lysis % of quadruplicate experimental samples minus the spontaneous release % (target cells incubated in medium alone) divided by the maximum release % (target cells incubated in centrimide) minus the spontaneous release %. Peptides were added to either the generation or effector phases of CTL development at a final concentration of 100 μg/ml. Toxic effects of the peptides were determined by the lysis % above background that was obtained by incubating labeled targets with peptide alone.

RESULTS AND DISCUSSION

Relative solvent accessibility (RSA) was calculated as the quantitative measure to evaluate the surface exposure of various CD8 loop regions. RSA was calculated as the solvent accessible surface area of an amino acid residue in a given loop region relative to its value in a tripeptide Ala-Xaa-Ala sequence (28). Table I lists the RSAaverage values for various sequences of CD8 surface loops. It was found that two loops, C’D and DE, have a RSAaverage value comparable with those of the CDR loops and thus may be possible sites for molecular interaction with MHC class I (Fig. 1).

To test the putative role of the CD8 C’D and DE loops, synthetic peptide analogs derived from these sites were synthesized. A disulfide bond was incorporated into these peptide sequences to form cyclic structures that could stabilize the peptide conformation mimicking the native loop region. The ability of the peptides in blocking CD8 interaction with MHC class I was measured in a cell adhesion assay. The cyclic DE loop peptide exhibited significant inhibitory activity (53%), whereas the cyclic C’D loop peptide did not show any effect, suggesting the involvement of the DE loop in binding to MHC class I molecules.

Presumably, the peptide exerts its inhibitory activity through the structural mimicry of a surface epitope of the native protein. Results from a molecular modeling study suggested that the cyclic DE loop peptide closely mimicked the conformational feature of the β-turn of the native DE loop (data not shown). To test if the β-turn structure of the CD8 DE loop is essential for the interaction with MHC class I, a linear DE loop peptide was synthesized and assessed. This linear DE loop peptide showed decreased activity in the inhibition of rosette formation as compared with the cyclic constrained analog (Fig. 2n), supporting the notion that the β-turn structure is important for the binding of the DE loop to MHC class I. An anti-CD8 monoclonal antibody was used as a positive control, which blocked CD8-MHC class I binding in the cell adhesion assay. As a negative control, a scrambled peptide (DE scr; identical amino acid composition, but a fixed randomized sequence) did not show any activity, demonstrating the sequence specificity of the activity of the DE loop peptide. To further characterize the amino acid residues and structural features within the CD8 DE loop important for its biological function, a series of DE loop peptide analogs containing alanine substitution at a single site were synthesized and their activity tested in the cell adhesion assay. As shown in Fig. 2a, the replacement by alanine at positions Leu-73, Asp-75, and Thr-76 led to substantial loss of activity, indicating that these residues located on the tip of the β-turn may form the bioactive core of the DE loop.

The CD8 DE loop peptide analogs were also tested for inhibitory effect on the in vitro function of CD8+ CTL in a CML assay. The cyclic DE loop peptide was shown to be highly effective in the blockade of specific lysis (82.1% inhibition) of target cells, whereas the linear DE loop peptide and peptide analogs containing alanine substitution, L73A, D75A, and T76A, exhibited decreased activities (Fig. 2b). These results were consistent with those observed in the cell adhesion experiments. Taken

![Image](92x515 to 255x729)

FIG. 3. The crystal structure of the CD8 DE loop (24), highlighting the Leu-73-Thr-76 region as the exposed MHC class I binding site and the potential interactions of Lys-71 with Val-78 and Arg-72 with Phe-77 important for the structural stability of the DE loop.
together, these findings clearly demonstrated the role of the CD8 DE loop in mediating CD8-dependent biological function and suggested the potential of CD8 DE loop peptides for therapeutic application. Studies are currently in progress in a murine skin allograft model, and preliminary data suggest that administration of the cyclized DE loop peptide can prolong graft survival.

Concomitant with the completion of the in vitro DE loop peptide studies, as described above, the crystal structure of the hCD8-MHC class I complex was recently reported (24). This allowed for an unbiased evaluation of the results about structure-function of the CD8 DE loop, which were obtained in the absence of this crystal structure. The crystal structure of the complex indicates that the hCD8 homodimer interacts with the α2, α3, and βm domains of MHC class I. Of particular note is the direct contact observed between the DE loop of CD8 and the DE loop of the βm domain of MHC class I (24). The CD8 DE loop serves as a distinct recognition site in addition to the interface between the CDR loops of CD8 and the α3 loop of MHC class I. This is in full agreement with the proposed role of the CD8 DE loop in mediating CD8 function, as suggested by our present study. The structure of the CD8-MHC class I complex also provides a basis to explain the activity of the DE loop peptide and its alanine substituted analogs, as observed in our study. In the crystal structure, the CD8 DE loop is highly exposed and serves as a direct contact site with MHC class I, particularly with Asp-75 located on the tip of the CD8 DE loop forming a hydrogen bond interaction with Lys-58 of MHC class I (24) (Fig. 3). This explains the substantial loss of activity in both cell adhesion and CML assays of DE loop peptide analogs (L73A, D75A, and T76A) with alanine substitutions around this critical region (Fig. 2).

The crystal structure of the CD8-MHC class I complex also suggests other residues important for the structural stability and biological function of the CD8 DE loop. As shown in Fig. 3, the aliphatic side chains of Lys-71 and Arg-72 seem to form potential hydrophobic interactions with the spatially proximate side chains of Val-78 and Phe-77, respectively. In addition, it is possible that the side chains of Arg-72 and Phe-77 may form a cation-π interaction (29). Such interactions may be critical for maintaining a stable β-turn conformation of the Leu73-Thr-76 region on the tip of the DE loop, which is responsible for the direct contact with MHC class I (Fig. 3). To test this hypothesis, we synthesized and tested four additional peptide analogs (K71A, R72A, F77A, and V78A) containing a single alanine substitution at various sites of the DE loop. All of these modifications resulted in the significant loss of biological activity of the DE loop peptide in both cell adhesion and CML assays (Fig. 2), thus strongly suggesting that these residues play an essential structural role for the function of the DE loop.

The results from this present study support the notion that small surface epitopes play a critical role in mediating large protein-protein interfaces (30). Although the interaction between CD8 and MHC class I, as revealed in the crystal structure of their complex, involves a large surface on both molecules with many contacting regions, the small peptide mimic of the CD8 DE loop was able to effectively inhibit the formation of the CD8-MHC class I complex as shown in the cell adhesion assay. Together with our earlier design and discovery of small peptide and nonpeptide inhibitors that block the stable association between CD4 and MHC class II proteins (4–6), these studies demonstrated an approach of using small molecules to modulate complex protein interactions in biological and pathological processes.

In summary, we have found that the DE loop is an important functional epitope on the hCD8 protein surface. Small synthetic peptide mimics of this site were able to effectively block hCD8-MHC class I cell adhesion and CD8+ CTL function in vitro. These results suggest that the DE loop region of the CD8 protein may represent a new target for the design and development of peptide and eventually nonpeptide immunoregulatory agents. Finally, it is important to note that the recently reported crystal structure of the CD8-MHC class I complex fully supports the original findings regarding the role of the CD8 DE loop obtained in the absence of the crystal structure. As such, this study may provide a critically tested example, following our previous study of CD4 peptide design (5, 7), for further demonstration of the concept and validity of bioactive peptide design based on protein surface epitopes. It is possible that such a strategy may serve as a general paradigm for the study of other protein-protein complexes, particularly those of IgSF molecules.

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