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Direct Binding of the MHC Class I Molecule H-2L^d to CD8: Interaction with the Amino Terminus of a Mature Cell Surface Protein

Marie T. Jelonek,* Brendan J. Classon,† Peter J. Hudson,‡ and David H. Margulies[1]*

MHC class I molecules (MHC-I) display peptides from the intracellular pool at the cell surface for recognition by T lymphocytes bearing $\alpha\beta$ TCR. Although the activation of T cells is controlled by the interaction of the TCR with MHC/peptide complexes, the degree and extent of the activation is influenced by the binding in parallel of the CD8 coreceptor with MHC-I. In the course of quantitative evaluation of the binding of purified MHC-I to engineered CD8, we observed that peptide-deficient H-2L^d (MHC-I) molecules bound with moderate affinity ($K_d = 7.96 \times 10^{-7}$ M), but in the presence of H-2L^d-binding peptides, no interaction was observed. Examination of the amino terminal sequences of CD8α and β chains suggested that H-2L^d might bind these protein termini via its peptide binding cleft. Using both competition and real-time direct assays based on surface plasmon resonance, we detected binding of empty H-2L^d to synthetic peptides representing these termini. These results suggest that some MHC molecules are capable of binding the amino termini of intact cell surface proteins through their binding groove and provide alternative explanations for the observed binding of MHC molecules to a variety of cell surface receptors and coreceptors. ***The Journal of Immunology,*** **1998, 160: 2809–2814.**

Both the activation of mature $\alpha\beta$ TCR-bearing T lymphocytes and the thymic maturation of precursor T cells are influenced by the intrinsic affinity of the particular TCR for MHC/peptide ligands and the interaction of the coreceptor molecules CD8 or CD4, which bind the MHC class I (MHC-I)[2] or class II molecules, respectively. In the case of MHC-I, adhesion and functional studies indicate that the nonpolymorphic α3 domain is the major focus of binding to CD8 (1–3). More recently, the behavior of a large set of CD8 mutants has been correlated with the three-dimensional structures of the CD8α homodimer and MHC-I in providing support for a model in which the CD8 Ig-like $\alpha\alpha$ homodimer interacts simultaneously with two MHC-I through both the α2 domain and α3 (4–6). Such a view is consistent with popular models in which multimerization of the TCR along with coreceptors is influenced by the coordinate multimerization of MHC/peptide complexes on the APC (7–9). However, the recent high resolution crystallographic structure of a CD8$\alpha\alpha$/HLA-A2 complex (10), which visualizes a single CD8$\alpha\alpha$ homodimer binding to a single HLA-A2 molecule through the α3 domain, suggests that parallel multimer formation may not be a function of CD8.

The initial descriptions of coreceptor binding to MHC-I were based on adhesion assays exploiting high CD8-expressing transfectant cells (2, 11, 12). However, it continues to be difficult to measure the interactions of purified preparations of MHC class I with purified CD8. Recombinant soluble CD8 has been coated onto plastic for monitoring the binding of MHC class I-bearing cells (13), and others have succeeded in binding soluble CD4 to cells (14). Recently, purified CD8 has been shown to facilitate the interaction of the TCR with MHC-I/peptide complexes in in vitro binding studies (15). This study complements recent examples in which the direct interaction of TCR with MHC/peptide complexes has been demonstrated (16–19). In addition, it provides further evidence that the contribution of CD8/MHC-I interaction to TCR binding is not merely static, but is influenced by a dynamic component, as suggested by cross-linking experiments (20).

To complement our previous studies on the relationship between the affinity of particular MHC/peptide complexes and the outcome of T cell activation, we have been analyzing the contribution of engineered forms of murine CD8 in binding to MHC-I and their contribution to the TCR/MHC/peptide interaction. In the course of assessing directly the binding of different preparations of the murine MHC-I H-2L^d for CD8$\alpha\alpha$ or CD8$\alpha\beta$ molecules, we observed that H-2L^d preparations lacking peptide exhibited direct binding while those tightly complexed with self-peptides or reloaded with synthetic peptides failed to bind. Here we explore this phenomenon and provide evidence that H-2L^d can bind the amino terminus of the CD8 chains with significant affinity.

Materials and Methods

Proteins and peptides

Soluble MHC-I were purified by immunoaffinity chromatography on mAb columns as previously described (21–24). The MHC-I sH-2L^d, sH-2L^d(sk), and control molecules sH-2D^d and sH-2K^b have been reported elsewhere, and consist of the α1 and α2 domains of the respective molecules linked to the α3 and carboxyl terminus of the obligately secreted MHC class I-like molecule Q10^b (sH-2L^d (25), sH-2K^b (26)), the α3 of H-2L^d and the carboxyl terminus of Q10^b (sH-2L^d (sk) (27)), or the α3 of sH-2D^d and carboxyl terminus of Q10^b (21, 28). In some experiments, the immunoaffinity-purified molecules were emptied of self-peptides by brief exposure to pH 12.5 followed by spin column size exclusion chromatography (24). Soluble CD8$\alpha\alpha$ comprising the mouse CD8α V-domain (residues 1–130) joined to the rat CD8α hinge peptide (residues 122–162) was expressed in Chinese hamster ovary cells as described (29). The soluble CD8$\alpha\alpha$ was purified

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[2] Abbreviations used in this paper: MHC-I, MHC class I molecules; RU, resonance units.

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Table I. *List of peptides used in this study*

Peptide Designation	Amino Acid Sequence									
pMCMV C4	Y	P	H	C	M	P	T	N	L	
p2Ca		L	S	P	F	P	F	D	L	
Qp2Ca	Q	L	S	P	F	P	F	D	L	
mCD8α peptides[a]										
5397 mCD8α-NT	K	P	Q	A	P	E	L	R	I	
5403	K	P	Q	A	P	E	L	R	I	C
5409	Q	A	P	E	L	R	I	F	C	
5415	Q	A	P	E	L	R	I	F		
mCD8 β peptides[a]										
5404 mCD8β-NTL	I	Q	T	P	S	S	L			
5405	L	I	Q	T	P	S	S	L	L	
5417	Q	T	P	S	S	L	L	V		

[a] CD8α-NT and CD8β-NT refer to the amino-terminal sequences of the mature mouse CD8α and CD8β chains, respectively.

from culture supernatant by ion exchange chromatography on DEAE Sephacel (Pharmacia, Uppsala, Sweden) followed by gel filtration on Sephacryl S-300 (Pharmacia). The OX8 mAb specific for the rat CD8α hinge sequence was used to monitor purification by ELISA. For soluble CD8$\alpha\beta$, a chimeric cDNA comprising the V-domain of mouse CD8β (codons 1–116 (30)) was joined, via two additional threonine codons, to the rat CD8α hinge peptide (codons 118–159). The construct was cotransfected with the CD8α cDNA into Chinese hamster ovary cells, which resulted in the production of a mixture of soluble CD8$\alpha\alpha$ and CD8$\alpha\beta$ molecules. Soluble CD8$\alpha\beta$ was resolved from soluble CD8$\alpha\alpha$ by immunoaffinity chromatography using the CD8β mAb 53-5.8 (31). mAbs were purified by protein A-Sepharose chromatography from cell culture supernatants. The mAbs anti-CD8α (53-6.72 and CT-CD8a) and anti-CD8β (53-5.8 and CT-CD8b) were purchased from PharMingen, San Diego, CA or from Caltag Laboratories, So. San Francisco, CA. sH-2L^d molecules contained available binding sites revealed by epitope induction assays with H-2L^d-binding peptides using mAb 30-5-7S (23), and were further analyzed by their ability to bind the cognate 2C TCR in the presence of appropriate peptide, (16, 19). The sH-2D^d preparations contained available peptide binding sites as demonstrated by epitope induction with H-2D^d-binding peptides using the mAb 34-5-8S (22), as well as by the ability to bind immobilized P18-I10-C7 (RGPGRACVTI) in the BIAcore (Pharmacia) (24, 32). sH-2K^b binding sites were confirmed by the ability of emptied molecules to bind to immobilized pOVA-C6 (SIINFCKL) (32, 33). Both sH-2D^d and sH-2K^b preparations were emptied of copurifying self-peptides and repurified as described elsewhere (32). All peptides, synthesized in the Laboratory of Molecular and Structural Biology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, as described (22, 23), were provided by Dr. J. Coligan, and are referred to by the single letter amino acid code (see Table I).

Real time surface plasmon resonance

All binding experiments were performed in a Pharmacia BIAcore 2000 at 25°C. Peptides with free thiol groups were coupled to the biosensor surface as described (24, 33). CD8$\alpha\alpha$ or $\alpha\beta$, and mAbs were immobilized with standard amine coupling procedures in 0.1 M sodium acetate at pH 5.1 or 6.0, respectively (16). Binding of purified MHC molecules to either peptide, mAb, or CD8-coupled surfaces was performed in HBST (20 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween-20). Peptide surfaces were regenerated by exposure to 50 mM phosphoric acid. Protein surfaces were regenerated by washout in HBST. Flow rates and other specific parameters are given in the figure legends.

Competition and epitope induction assays

Purified sH-2L^d was analyzed for direct binding to immobilized CD8 either alone or following incubation with graded concentrations of the indicated peptides for 30 min at ambient temperature. Competition curves and kinetics association and dissociation binding curves were analyzed by curve fitting to appropriate expressions for the simple Langmuirian reaction A + B $\leqq$ AB as described in detail elsewhere (16, 19). Binding of peptides to H-2L^d was evaluated by either competition or epitope induction assays. Epitope induction assays were performed on emptied preparations of sH-2L^d, which were exposed to graded concentrations of the test peptides and then passed over a biosensor surface coupled with the α1α2 domain-specific conformationally dependent mAb 30-5-7S, as described (23). Competition binding, in which soluble peptides were used to inhibit the interaction of sH-2L^d with immobilized indicator peptides, was conducted as described (24, 32). MHC-I were incubated with different concentrations of competing peptides at ambient temperature for 30 min before the binding assay.

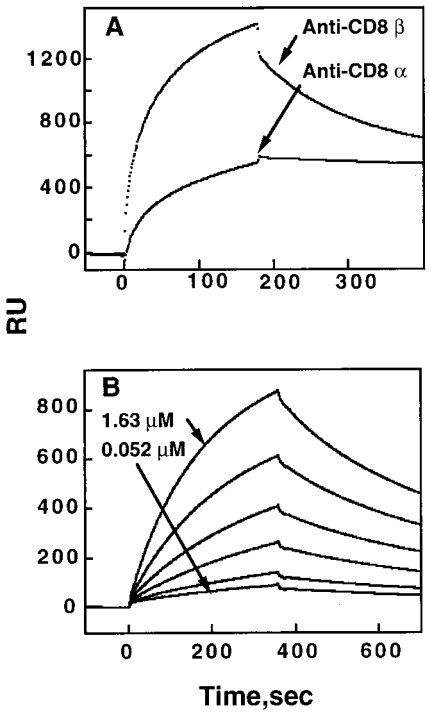

FIGURE 1. Binding of sH-2L^d to immobilized CD8. CD8$\alpha\beta$ was coupled to a biosensor surface as described in *Materials and Methods* to a level of 4376 RU. *A*, Binding of anti-mouse CD8α (0.5 μM) and anti-mouse CD8β (0.67 μM). The binding phase was initiated at $t = 0$, and a buffer washout phase started at $t = 180$ s. *B*, Concentration dependence of binding of an emptied preparation of sH-2L^d(sk) to a CD8$\alpha\beta$ surface. Sensorgrams of doubling dilutions from 1.63 to 0.052 μM are shown. The binding phase begins at $t = 0$, washout at $t = 360$ s.

Results

Binding of H-2L^d to mouse CD8

To assess quantitatively the interaction of purified CD8 and MHC-I, we explored the use of surface plasmon resonance detection using covalently immobilized murine CD8 and solution phase soluble MHC-I. Surface plasmon resonance measures the local changes in macromolecular concentration due to the binding of a solution phase ligand to an immobilized receptor. For illustrative purposes, we show the results for CD8$\alpha\beta$ coupled to the biosensor surface (Fig. 1). mAbs against both CD8α and CD8β bind CD8, as revealed by the time-dependent increase in resonance units (RU), indicating the preservation of the epitopes after chemical coupling (Fig. 1*A*). Using a preparation of soluble H-2L^d as the solution phase ligand, we observed the time- and concentration-dependent interaction with the immobilized CD8 (Fig. 1*B*). Although we expected this to be a relatively low affinity interaction, characterized by rapid dissociation, inspection of the washout portion of these binding curves indicated that this was tighter binding than expected, with a k_d (kinetic dissociation rate constant) of $1.72 \times 10^{-3} \pm 5.25 \times 10^{-6}$ s^{-1}. This result was observed with all preparations of soluble H-2L^d, although the degree of binding to CD8 appeared to be related to the peptide binding capacity of the particular preparation. In addition, other soluble, emptied MHC-I

H-2K^b and H-2D^d, which copurify with a higher level of occupancy of their peptide binding clefts, did not bind the CD8 molecules, irrespective of whether they contained a bona fide class I α3 domain or that of the class Ib molecule Q10^b (data not shown).

To determine whether the observed binding was influenced by the occupancy of the MHC molecule with peptide, we tested the binding of soluble H-2L^d to the immobilized CD8 in the presence of peptides known to bind well to H-2L^d, the viral peptide derived from the murine CMV pp89 immediate early protein, pMCMV (YPHFMPTNL) (34), and the self-peptide derived from α-ketoglutarate dehydrogenase, p2Ca (LSPFPFDL) (35). These peptides profoundly inhibited the binding of H-2L^d to CD8 (data not shown).

These data suggested the hypothesis that H-2L^d interacts with CD8 either directly through its peptide binding cleft, or through another site of H-2L^d that is available only in the peptide-free state. Inspection of the amino acid sequences of the amino termini of the mature mouse CD8α- and CD8β-chains (see Table I) indicated that CD8α contained a good candidate peptide for the known H-2L^d-binding motif, XPXXXXXX(L/I) (23), KPQAPELRI. This CD8α N-terminal peptide (5397; Table I) and several other peptides corresponding to the amino terminus of CD8β and adjacent regions were synthesized and tested in several ways for their ability to bind sH-2L^d and to inhibit the binding of sH-2L^d to CD8.

Binding of the CD8-derived peptides was compared with that of other H-2L^d-binding peptides using an assay in which solution phase peptides compete for binding of H-2L^d to the immobilized pMCMV-C4 peptide coupled to a biosensor surface (Fig. 2). The known H-2L^d-restricted self-peptide, Qp2Ca (QLSPFPFDL) (36), was used as a positive control, and showed half-maximal inhibition of binding at a concentration (ID_{50}) of about 0.03 μM. Peptide 5397, representative of the amino-terminal nine residues of CD8α (see Table I), as well as 5403, the same peptide with an additional carboxyl-terminal cysteine, competed effectively for the binding of H-2L^d to the immobilized pMCMV-C4 peptide, with ID_{50} values of 0.62 and 4.9 μM, respectively. In addition, peptide 5404, representative of the amino-terminal nine residues of CD8β, also bound, but relatively poorly (ID_{50} = 52 μM). (As a negative control, a peptide representing the amino-terminal 11 amino acids of rat CD8α with an added cysteine, QLQLSPKKVDAC, failed to show any inhibition of binding even at a concentration as high as 500 μM (data not shown).) The other peptides examined, 5405 and 5417, representing residues 1 to 9 and 3 to 10 of mouse CD8β, as well as 5409 and 5415, representing residues 2 to 9 of mouse CD8α, all showed significant binding to H-2L^d with a range of ID_{50} values from 1.0 (peptide 5405) to 110 μM (peptide 5417).

Binding as measured by such displacement curves may reflect direct competition at the binding site or may indicate some more complex conformational mechanism. Epitope induction, which measures the appearance of a new site as indicated by binding to a specific mAb, would add support to the view that these peptides are indeed binding to H-2L^d in a way that confers the conformation detected by the particular mAb. Figure 3 illustrates the results of such an assay using Qp2Ca as the positive control peptide. Thus, at high concentrations (100 μM), all these peptides cause H-2L^d to increase its ability to bind the mAb 30-5-7S, suggesting that they all bind in the peptide binding site.

The above data establish that peptides representative of the amino terminus of either the CD8α- or CD8β-chain can bind H-2L^d. We then asked whether the same peptides that bind H-2L^d would inhibit the interaction of H-2L^d with immobilized CD8$\alpha\beta$. As shown in Figure 4, *A* and *B*, soluble H-2L^d binding to CD8$\alpha\beta$ was inhibited by either the 5397 peptide (amino-terminal nonamer of CD8α) (see Fig. 4*A*), or the 5405 peptide (amino-terminal nonamer of CD8β) (see Fig. 4*B*). Significant inhibition of binding was effected by these peptides at concentrations from 0.1 to 1.0 μM, and almost total inhibition by 100 μM. Binding was not inhibited by peptides that fail to bind H-2L^d (data not shown). Thus, the direct binding of H-2L^d to CD8 is specifically inhibited by peptides that bind the MHC binding cleft.

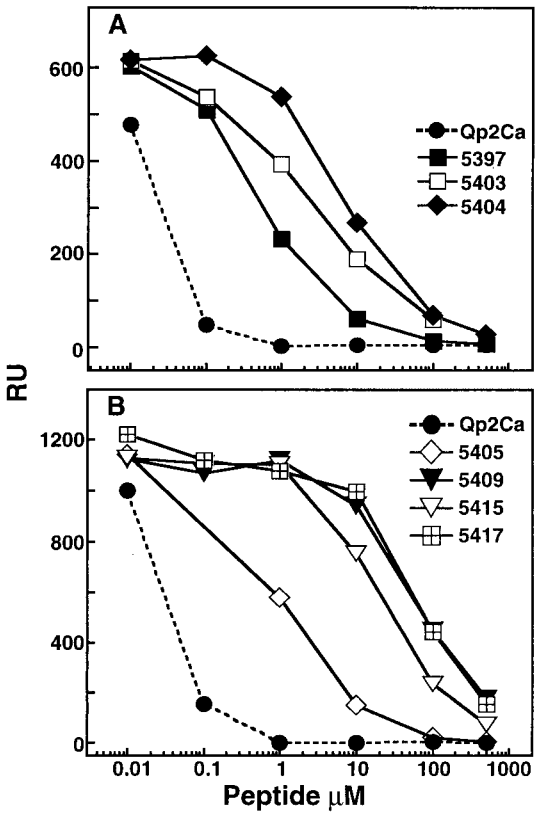

FIGURE 2. Mouse CD8 peptides compete for binding to sH-2L^d. *A*, pMCMV C4 was covalently coupled to a biosensor chip to a level of 20 RU as described in *Materials and Methods*, and binding of sH-2L^d(sk), emptied of self-peptides, at a concentration of 0.083 μM was assessed with preincubation of the indicated peptides as described in *Materials and Methods*. Peptide sequences are given in Table I. The binding, expressed as RU, at 5 min is indicated. Solution phase ligand was offered at a flow rate of 10 μl/min. Similar results were obtained for different preparations of sH-2L^d or sH-2L^d(sk), and the same rank order of peptide binding was obtained even when unemptied preparations were used. *B*, Data for the indicated peptides was obtained in the same experiment as for *A*.

The ability of an MHC-I such as H-2L^d to bind a peptide in solution does not necessarily indicate that that MHC molecule can bind that peptide sequence in its native configuration when linked via its carboxyl terminus to the rest of the mature protein. This is particularly true of MHC-I, which clearly have a preference, and usually a requirement, for the free carboxyl terminus of the peptide. To assess the ability of solution phase H-2L^d to bind directly, we coupled peptide 5403, representing the amino terminus of CD8α, through its carboxyl-terminal cysteine side chain, to the biosensor surface, and measured binding of soluble H-2L^d. As shown in Figure 4*C*, soluble H-2L^d clearly bound to this peptide even though the peptide had been coupled through its carboxyl-terminal residue. This binding is characterized by a rapid dissociation phase (k_d = 0.13 s^{-1}), and by inhibition by H-2L^d-binding peptides.

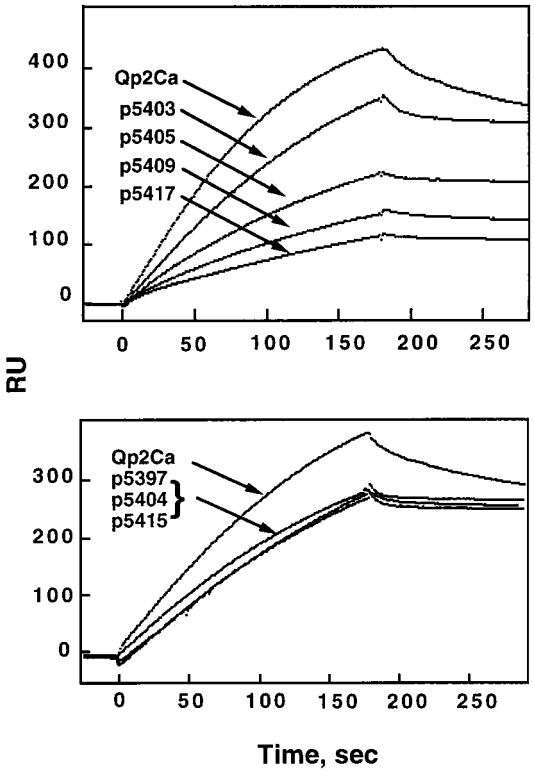

FIGURE 3. CD8-derived peptides increase the ability of H-2L^d to bind mAb 30-5-7S. H-2L^d (0.13 μM) in the presence of 100 μM of the indicated peptide was exposed at $t = 0$, to a biosensor surface coupled to mAb 30-5-7S (2795 RU).

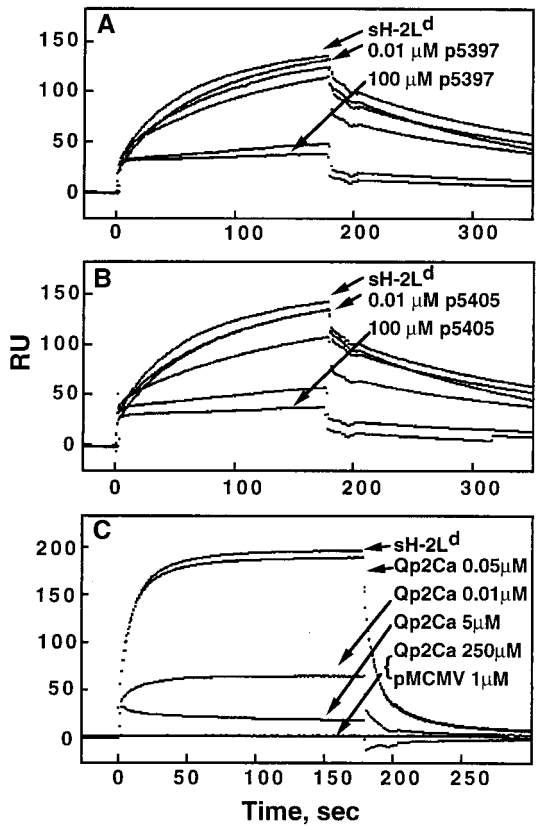

FIGURE 4. Binding of H-2L^d to CD8 and an immobilized CD8-derived peptide. CD8$\alpha\beta$ was covalently coupled to a biosensor surface as indicated in *Materials and Methods* (*A* and *B*) and exposed to solution phase soluble H-2L^d in the presence of graded concentrations (10-fold dilutions) of either peptide 5397 (*A*) or 5405 (*B*). For the experiment shown in *C*, peptide 5403 was coupled to the biosensor surface by thiol chemistry as described in *Materials and Methods*, and was exposed to soluble H-2L^d (3.0 μM) in the presence of the competing peptides Qp2Ca or pMCMV.

Discussion

Using engineered soluble forms of CD8$\alpha\beta$ and H-2L^d, we have demonstrated here that emptied H-2L^d molecules bind intact CD8 through the amino terminus of the CD8 protein. The apparent affinity of this protein-protein interaction is relatively high (k_d = 7.96×10^{-7} M; $k_a = 2.16 \times 10^3$ M^{-1} s^{-1}; $k_d = 1.72 \times 10^{-3}$ s^{-1}), and this complex is much more stable than the complex formed between H-2L^d and the amino terminal peptide of CD8α (k_d = 0.13 s^{-1}). The low k_a suggests that rare conformations of the MHC or of the CD8 may be required for this binding. Since peptides derived from both the CD8α and CD8β chains effectively bind H-2L^d and compete for the binding of H-2L^d to CD8, it is likely that H-2L^d can interact with CD8 through the amino termini of both chains.

The conventional view of the interaction of MHC molecules with TCR and with coreceptors is that the MHC/peptide complex is a globular trimer of the MHC heavy chain, light chain, and the assembled peptide. This heterotrimer binds by surface/surface interactions with a binding site formed by the juxtaposed CDRs 1, 2, and 3 of the TCR Vα and Vβ domains (37–39). The "physiologic" interaction with CD8 is thought to focus the CD8 Ig-region dimer on the MHC α3 domain (4), with additional CD8 contact to α2 (5, 6). The results we report here indicate that the empty binding groove of H-2L^d can bind the mature CD8 protein, peptides derived from the amino termini of both CD8α and CD8β, and a CD8α peptide covalently immobilized through its carboxyl terminus to a solid phase.

The three-dimensional structure of the human CD8$\alpha\alpha$ homodimer reveals an Ig variable (Ig-V) domain fold (4). Residues 2 through 7 (which in alignment correspond to mouse CD8α residues 6 through 11) form the first strand of β sheet that is H-bonded to residues 21 through 25. The mouse CD8α has four residues at the amino terminus that extend beyond the amino terminus of human CD8 and would be expected to be accessible to solvent and thus to MHC-I. Of course, slight unfolding or mild denaturation of the mouse CD8 cannot be completely ruled out, but we have consistently observed the binding to H-2L^d irrespective of the particular preparation of CD8 used. Leahy, Axel, and Hendrickson noted that the N-terminal strand of human CD8α was in an unusual main-chain configuration (not V_L-like) and speculated that the N-terminal strand could be flexible and potentially rearrange between V_L and CD8 conformations (4).

The major question raised by these observations is whether this MHC class I/CD8 interaction is of physiologic importance or whether this is merely a binding curiosity. Our own efforts, using MHC-I H-2D^d and H-2K^b molecules emptied of copurifying self-peptides by treatment at high pH and repurification, have failed to demonstrate the direct binding of these molecules to the amino termini of CD8 (data not shown). However, from a technical standpoint, our observations raise the possibility that the interaction of at least some MHC molecules with surface molecules on APCs can occur through molecular mechanisms other than the classical one in which a tight MHC/peptide complex as a unit interacts with a binding site formed by a protein surface (such as that of the TCR-$\alpha\beta$ or of CD8). The possibility must be considered that MHC

molecules free of self- or antigenic peptides are capable of interacting with the N terminus of mature proteins on the APC. Although the dogma for MHC class I peptide binding is that the side chain of the carboxyl-terminal amino acid is critical for binding to the F pocket, and the presence of the carboxyl-terminal carboxylate is necessary for stabilization through salt bridges to basic side chains of the MHC such as the conserved lysine of position 146 of the MHC-I heavy chain (22, 40), there are several examples that indicate that MHC molecules do not have an absolute requirement for a free carboxyl terminus (41, 42).

In addition, a number of reports of MHC interactions with other cell surface receptors, such as the insulin receptor (43–49), CD8 in *cis* on T cells (50), IL-2 receptor (51), luteinizing hormone receptor (52, 53), β-adrenergic receptor (53–55), epidermal growth factor receptor (56), and muscarinic cholinergic receptor (57) have been reported. All of these interactions occur in *cis* between MHC class I and the other membrane receptor. It is provocative to note that the amino-terminal sequence of the human insulin receptor β-chain (SLGDVGNVT) as well as that of the amino-terminal 10-mer of human CD8β (LQQTPAYIKV) score high when analyzed by a computer program (58) for their stability to bind to the most common human MHC class I allele, HLA-A0201. Some of these associations have well-documented physiologic consequences, while others may reflect associations the biologic significance of which is yet to be determined. Since there is the potential of empty surface MHC-I to interact with other mature molecules both in *cis* and *trans*, it appears that the cellular expression system has devised multiple strategies to minimize the escape of empty MHC-I to the cell surface, including requirements for peptide, glycosylation, β_2-microglobulin, various chaperonins, tapasin, and transporter associated with Ag processing (59–67). As these are important aspects of the normal biosynthesis of MHC-I, and since interactions with tapasin, calnexin, calreticulin, and transporter associated with Ag processing have only been incompletely localized, it will be worthwhile to consider these in light of the possibility of interaction through the MHC-I binding groove.

The recent application of direct binding methods in the assessment of the contribution of CD8 to the formation of a TCR/MHC/peptide ternary complex has important implications in our understanding of the initial events in TCR-mediated cellular activation (15). Our work raises a cautionary note: any interaction between an MHC molecule and another protein must also be evaluated with respect to the possibility that empty MHC molecules might bind through unorthodox mechanisms; that is, the binding groove may bind the amino termini of the mature protein. Although we have not detected such binding with the limited number of other MHC-I that we have tested, this mode of interaction may be characteristic of MHC-I that tend to have a loose association with peptide (such as H-2L^d or HLA-B27) and that therefore may be more promiscuous in their interactions with tethered peptides. Whether such interactions reflect the physiologic function of these molecules will require further experimentation.

Acknowledgments

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