Enhanced Immune Presentation of a Single-chain Major Histocompatibility Complex Class I Molecule Engineered to Optimize Linkage of a C-terminally Extended Peptide

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Major histocompatibility complex (MHC)† class I molecules are heterotrimers consisting of a transmembrane (type I) heavy chain, a soluble light chain (β₂-microglobulin (β₂m)), and a peptide ligand of typically 8–10 amino acids. The initial assembly of the class I heterotrimers occurs in the endoplasmic reticulum (ER) and requires the concerted activity of several accessory molecules. Complete assembly of the heterotrimer is necessary for ER egress and stable surface expression of class I molecules (1, 2). On the cell surface, class I molecules containing the appropriate peptides can mark pathogen-infected or malignant cells for elimination by cytolytic CD8 T lymphocytes (CTL). As clear evidence of the importance of class I antigen presentation, numerous virus-encoded proteins that block various steps in the class I assembly pathway to evade host immune responses have been identified (3).

Studies of the structure and function of several different oligomeric immune receptors have been facilitated by the covalent attachment of various subunits via flexible linkers. This is particularly true in the case of MHC class II molecules. The peptide ligand-binding site of MHC class II molecules, in contrast to class I molecules, is formed by the interaction of two separate membrane-bound chains designated α and β. To produce MHC class II molecules occupied by homogenous peptides, constructs were made whereby different peptides were attached to the N terminus of the class II β-chain using flexible linkers (4). Class II molecules with covalently bound single peptides have been very informative in studies of class II assembly and importance of chaperone molecules in optimal class II peptide loading (4–6). Furthermore, such class II molecules with covalently attached peptides have provided insights into CD4 T cell development and the specific role of the peptide bound to class II (7).

Based on these successes, it would be very attractive to tether a peptide to an MHC class I molecule. In the case of the class I molecule, the ligand-binding site is formed between two outer domains (α₁ and α₂) of the class I heavy chain in a manner independent of the heavy chain α₂-domain (8). Whereas the structure of the class II molecule accommodates peptides protruding from the ends of its ligand-binding groove, the class I structure optimally accommodates peptides of defined length that do not extend beyond the binding groove (9, 10). In fact, interactions between the peptide termini and conserved class I heavy chain residues at the ends of the binding groove are critical for stable peptide anchoring (11). Therefore, a linker that extends from the C-terminal residue of the peptide might be predicted to interfere with strong peptide binding. Indeed, attempts to engineer class I heavy chains with tethered peptides at the N terminus have been successful only for select peptide-class I complexes (12, 13).² By contrast, linking β₂m to the heavy chain using a flexible linker appears to be universally applicable for different mouse and human class I molecules (14–16). More recently, others have produced constructs with the peptide covalently attached to free β₂m (17–
Although MHC class I heavy chains (H-chains) can associate with these peptide-β2m molecules and present the peptides to T cells, it remains unclear the extent to which these molecules exclude the binding of competing free peptide ligands.

Due to the limitations described above, we (21) and others (20, 22) have investigated strategies to produce completely assembled class I molecules whereby all three components (heavy chain, β2m, and peptide) are attached by flexible linkers. By ordering the components in the format peptide-spacer-β2m-spacer-heavy chain and by optimizing linker lengths, we were able to produce single-chain trimers (SCTs) of class I. This format appears to be widely applicable for different mouse and human H-chains and their respective peptide ligands (20–22).

We have focused our initial characterizations on the K\(^b\) SCT containing the ovalbumin peptide (OVA) due to the availability of a three-dimensional structure and well characterized peptide-specific monoclonal antibodies (mAbs) and T cells (23–25).

Thus far, our investigations have revealed that antibodies and T cells recognize cognate SCTs very similar to normal class I molecules bound with noncovalently attached peptides (referred to here as native class I molecules). Furthermore, SCTs display rapid assembly in the ER and have very stable expression at the cell surface, properties consistent with their covalent nature (21) (data not shown). As a likely reflection of their surface stability, we have shown that the OVA-β2m-K\(^b\) SCTs are potent stimulators of antibodies and CTL specific for native OVA-K\(^b\) peptide complexes (21). The surface stability of SCTs was surprising in light of the aforementioned notion that the class I binding groove appears to be “closed” and the C terminus of the peptide interacts with conserved H-chain residues prominent in anchoring the peptide. However, it is noteworthy that peptides with C-terminal extensions have been eluted from class I heavy chains isolated with unique antibodies (26, 27). Furthermore, the structure of a class I molecule with a C-terminally extended peptide suggested a potential mechanism to accommodate such extensions through unique arrangements of heavy chain residues (28). Thus, we considered it important to probe the mechanism of peptide binding by SCTs to better exploit them for future applications, including the in vitro and ex vivo stimulation of T cells to treat human diseases.

In this study, we explore immunological recognition of SCTs that have been engineered with a mutation of the heavy chain residue Tyr\(^{22}\), a conserved residue that creates part of the F pocket of the peptide-binding groove. We further compare the accessibility of SCTs with and without this mutation for binding exogenous free peptides. Our findings suggest that the enhanced surface stability of SCTs is rendered by their ability to continuously rebind the attached peptide. In addition, we report the production of recombinant SCT molecules and their use to generate tetramers for enumeration of antigen-specific T cells. This latter finding may result in improved tetramers for peptide-class I peptide complexes that are prone to dissociation.

**EXPERIMENTAL PROCEDURES**

**Mice—**OT-1 T cell receptor transgenic mice (H-2\(^d\)) contain the rearranged transgenes encoding the T cell receptor specific for K\(^b\) SIINFEKL (23) on a RAG-1\(^{–/–}\) background and were kindly provided by Dr. Skip Virgin (Washington University School of Medicine), and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME).

**Cell Lines, Antibodies, and Peptides—**L cell line ML1.8 (H-2\(^d\)) (29) was a gift from Dr. Phillipe Kourilsky (INSERM, Institut Pasteur, Paris, France). The mAbs used in this study include the following: 64-3-7, which recognizes open (peptide-free) forms of class I molecules tagged with this epitope (30); BR-24, which recognizes folded K\(^b\) (American Type Culture Collection, Manassas, VA); 25-D1.16 (a gift of Dr. Jonathan Yewdell, National Institutes of Health), which recognizes K\(^b\) + the SIINFEKL peptide (24); and YS, which recognizes folded H-2\(^d\) molecules (31). The OVA-derived peptide (SIINFEKL) (32) and SIYR peptide (SIYRYYGL) (33) were synthesized on an Applied Biosystems Model 432A peptide synthesizer.

**DNA Constructs—** Constructs were generated using standard techniques and were confirmed by DNA sequence analysis. The generation of mAb 64-3-7 epitope-tagged K\(^b\) (K\(^b\) R48Q/R50P) and the OVA-β2m-K\(^b\) (OVA-K\(^b\)) SCT has been described (21). The OVA-K\(^b\) SCT consists of, beginning at the N terminus, the leader sequence of β2m followed immediately by the SIINFEKL sequence and then a linker of 15 residues ([G\(_6\)S\(_8\)]). This first linker is followed by the mature β2m sequence, the second linker of 20 residues ([G\(_6\)S\(_8\)]), and then the mature K\(^b\) sequence. The Y84A mutation in K\(^b\) and the OVA-K\(^b\) SCT was created using PCR mutagenesis. Constructs were expressed from the pIRESmneo vector (Clontech). For expression of SCT constructs in Escherichia coli, the insertion sequence was replaced by a methionine in the N terminus upstream of the SIINFEKL sequence. Furthermore, the K\(^b\)-H chain (without the 64-3-7 epitope) was truncated at residue 280, just upstream of the transmembrane domain, and a BirA biotinylation sequence (GGGLNDIEAFLKIEWHE) was added. These constructs were expressed from the pET21a vector (Novagen, Madison, WI).

**CTL—**The OVA-K\(^b\) (SIINFEKL) CTL were generated from OT-1 mice. Freshly explanted splenocytes (2.5 × 10\(^6\)) were cultured with 2.5 × 10\(^6\) irradiated splenocytes (2000 rads) in the absence of interleukin-2 and in the presence of 5 × 10\(^–6\) M SIINFEKL peptide. After 5 days, the cells were harvested and used in a 2\(^5\)Cr release assay. Target cells (2–3 × 10\(^6\)) were labeled for 1 h with 150 μCi of 51Cr (Na\(^{51}\)CrO\(_4\); New England Nuclear Life Sciences). Cells were washed twice and were plated onto 96-well microtiter plates, and washed target cells (2 × 10\(^5\)) were added. For some groups, the SIINFEKL peptide was present during the assay at a final concentration of 5 μM. The plates were centrifuged at 50 × g for 1 min and were incubated for 4 h at 37 °C in 5% CO\(_2\). Radioactivity in 100 μl of supernatant was measured in an Isomadic γ-counter (ICN Biomedicals, Huntsville, AL). The mean of triplicate samples was calculated, and percentage 51Cr release was determined according to the following equation: % 51Cr release = 100 × ((experimental 51Cr release − control 51Cr release)/(maximum 51Cr release − control 51Cr release)), where experimental 51Cr release represents counts from target cells mixed with effector cells, control 51Cr release represents counts from target cells in medium alone, and maximum 51Cr release represents counts from target cells lysed with 5% (v/v) Triton X-100 (Sigma).

**Production of Recombinant Proteins and Tetramers—** Recombinant OVA-K\(^b\) and OVA-K\(^b\) Y84A SCTs were produced as insoluble inclusion bodies following induction with 1 mM isopropyl-β-D-thiogalactopyranoside in E. coli BL21(DE3) Codon Plus RIL cells (Stratagene). These inclusion bodies were purified and then dissolved in 6 M guanidine, 10 mM Tris (pH 8.0), and 20 mM β-mercaptoethanol. N-terminal sequencing of each of these proteins revealed that the N-terminal Met residue had been removed during expression in E. coli. Protein refolding was accomplished using a standard MHC class I refolding protocol. Briefly, three injections of protein, each of which raised the total concentration of protein in refolding buffer (400 mM t-arginine, 100 mM Tris (pH 8.0), 2 mM NaEDTA, 0.5 mM oxidized glutathione, and 5 mM reduced glutathione) by 1 μM, were added over 3 days to slowly stirred refolding buffer at 4 °C. Refolding reactions were concentrated and subjected to gel filtration chromatography using a Superdex 200 16/60 column (Amersham Biosciences) and in vitro biotinylated for 12 h at 20 °C in the presence of 15 μg of BirA (Avidity, Boulder, CO), 80 μM biotin, 100 mM ATP, 10 mM MgOAc, 20 mM Bicine, and 10 mM Tris-HCl (pH 8.3). To remove free biotin, monomeric complexes were again purified by gel filtration, tested for biotinylation by Western blotting, and then tetramerization by addition of phycoerythrin-labeled streptavidin (Molecular Sciences) at a molar ratio of 4 molecules of SCT monomers to 1 molecule of phycoerythrin-labeled streptavidin.

**Enzyme-linked Immunosorbent Assay (ELISA)—** Purified recombinant OVA-K\(^b\) and OVA-K\(^b\) Y84A SCTs were bound to Nunc Maxisorp immunoplates (Nalge Nunc, Rochester, NY) overnight at 4 °C in phosphate-buffered saline (pH 7.2) containing 1% 1,4-β-D-thiogalactopyranoside and 2 μg/ml of each of these proteins were added. Plates were incubated at 4 °C for 1 h to block the wells and were then washed three times with phosphate-buffered saline. Absorbance at 450 nm was measured using a Bio-Rad Model 550 plate reader.
analyzed using CellQuest software (BD Biosciences). Staining with anti-class I mAbs was visualized using phycoerythrin-conjugated goat anti-mouse IgG (BD Biosciences). For tetramer analysis, cells were stained concurrently with fluorescein isothiocyanate-labeled anti-CD8 antibody (Caltag Laboratories, Burlingame, CA) and phycoerythrin-labeled tetramers (conventional or SCT-based). Conventional OVA-Kb tetramers (noncovalent) were obtained from the NIAID MHC Tetramer Core Facility (Atlanta, GA). Brefeldin A (BFA) turnover was performed as described (34).

RESULTS

Engineering the Class I Binding Groove for Greater Tolerance of the C-terminal Peptide Extension—Our initial characterization of OVA-Kb SCTs demonstrated that, although they exhibited substantial reactivity with an antibody (25-D1.16) specific for OVA-Kb complexes (24), they did not react quite as well as native Kb fully loaded with exogenous OVA (Fig. 2 and Table I). Because this mAb is known to interact with the C-terminal region of OVA when bound to Kb (35), we suspected that the presence of the linker in the SCT might decrease the efficiency of binding by this mAb. Therefore, we sought to introduce a mutation into the class I H-chain that would render it more tolerant to the linker extending from the C terminus of the peptide to \(\mu_m\) (Fig. 1A). In this regard, inspiration was taken from MHC class II proteins. They have evolved a peptide-binding groove that is structurally similar to MHC class I proteins with the exception that it is truly open at both ends, allowing it to bind much longer peptides in an extended conformation throughout its entire peptide-binding groove. Taking advantage of this attribute, many different MHC class II molecules with covalently linked peptides have been produced and crystallized. Comparison of the peptide-binding groove from OVA-Kb (25) with the peptide-binding groove from the covalently linked MHC class II I-E\(^{k}\)-HLA\(^{\alpha}\) complex (36, 37) suggested that Tyr\(^{84}\) in Kb plays a major role in closing the peptide C-terminal end of the binding groove. Tyr\(^{84}\) is an invariant residue in MHC class Ia molecules and is essential for peptide binding stability (10). As shown in Fig. 1B, Tyr\(^{84}\) contributes in a significant way to the formation of the F pocket, where it creates the C-terminal “wall” and helps sequester the peptide C terminus via hydrogen bonding. The crystal structure of HLA A2 in complex with a decamer peptide from calreticulin demonstrated the effectiveness of this Tyr\(^{84}\) wall, as the C-terminal glycine of the peptide was forced to turn \(90^\circ\) and to project away from the plane of the binding groove directly into solvent (28).

We speculated that mutation of Tyr\(^{84}\) to alanine would partially open the binding groove and permit a peptide C-terminal linker to adopt a more extended conformation. As detailed below, this mutation did improve recognition of the OVA-Kb SCT by peptide/MHC-specific mAbs and T cells and, more importantly, provided insights into the mechanism by which SCTs maintain their stable structure.

Native Kb H-chains with the Y84A Mutation Exhibit Relatively Poor Peptide Binding—To explore the importance of Tyr\(^{84}\) for peptide binding, we introduced the Y84A mutation into Kb and the OVA-Kb SCT. In addition, to better assess the quality of peptide binding, the epitope recognized by mAb 64-3-7 was also introduced into all of the constructs. We have shown previously, with several different mouse and human class I molecules (including Kb), that this epitope tag permits the discrimination of peptide-occupied (folded) versus peptide-empty (open) H-chain conformers without altering the peptide binding preferences of each respective molecule (30, 34, 38, 39).

Initially, we compared the quality of peptide binding between native Kb (no covalent attachments) versus Kb Y84A molecules expressed on L cells (H-2\(^{b}\)). This was accomplished serologically using mAb B8-24-3 to detect peptide-occupied surface Kb and mAb 64-3-7 to detect peptide-empty surface Kb. Using this approach, we estimated that 21% of native Kb molecules were peptide-empty at steady state, whereas 89% of Y84A Kb molecules were peptide-empty (Fig. 2A, upper panels, and Table I). Given that peptide occupancy by class I is a requirement for ER exit (1, 2), these data imply that the Y84A mutation renders Kb molecules less able to retain their bound peptides. Consistent with this idea, overnight incubation of Y84A mutant molecules with exogenous OVA peptide substantially increased surface expression of folded molecules (3.6-fold increase), whereas native Kb exhibited only modest induction (1.3-fold) (Fig. 2A and Table I). This indicated that exogenous peptides could more easily displace the peptides acquired by the Y84A mutants in the ER. Together, these results confirm that Tyr\(^{84}\) plays an important role in peptide binding to the H-chain.

Effects of the Y84A Mutation on the SCT Background—We next performed a similar comparison of OVA-Kb SCTs with and without the Y84A mutation. As shown in Fig. 2B and summarized in Table I, the OVA-Kb SCT displayed very few peptide-empty forms at the cell surface (1–2%), regardless of the presence of the Y84A mutation. The paucity of peptide-empty forms of the Y84A SCT molecules contrasted with the poor peptide occupancy we observed for native Kb with the Tyr\(^{84}\) mutation (Fig. 2A). This disparity indicates that having the peptide covalently attached in the SCT overcomes the otherwise detrimental effects of the Y84A mutation.

Comparison of the wild-type versus Y84A SCT molecules using the OVA-Kb-specific mAb (25-D1.16) revealed that this mutation improved the interaction of mAb 25-D1.16 with the SCT. mAb 25-D1.16 recognizes the OVA-Kb complex (36, 37) specifically using mAb B8-24-3 to detect peptide-occupied surface Kb and mAb 64-3-7 to detect peptide-empty surface Kb. Using this approach, we estimated that 21% of native Kb molecules were peptide-empty at steady state, whereas 89% of Y84A Kb molecules were peptide-empty (Fig. 2A, upper panels, and Table I). This indicated that exogenous peptides could more easily displace the peptides acquired by the Y84A mutants in the ER. Together, these results confirm that Tyr\(^{84}\) plays an important role in peptide binding to the H-chain.

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membrane domain.

SCTs with Y84A Are Stable at the Cell Surface—The above findings indicate that the Y84A SCT molecules retain binding of the covalent peptide and, in fact, appear to better tolerate the linker between the peptide and βm. To determine whether this mutation affects the stability of the molecules, their cell-surface turnover was analyzed in the presence of BFA. BFA blocks the secretory pathway to prevent the arrival of newly synthesized class I molecules at the cell surface, permitting a determination of the half-life of pre-existing surface molecules (40, 41). We have previously shown that OVA-Kb SCTs are more stable under these conditions than native OVA-Kb complexes (21). Here, cells expressing Kb, Kb loaded with exogenous OVA peptide, or SCT or Y84A SCT molecules were treated with BFA, and their respective turnover rates were monitored by flow cytometry. As shown in Fig. 3, SCTs with or without the Y84A mutation were remarkably stable at the cell surface. Whereas normal Kb had a half-life of ~8 h, both SCT forms displayed little turnover during the 16-h time course (as detected with either mAb B8-24-3 or 25-D1.16). Furthermore, as indicated by the mAb 25-D1.16 staining, both SCTs were more stable than Kb molecules fed the OVA peptide overnight. Thus, the Y84A mutation did not affect the remarkable cell-surface stability of the SCTs, consistent with their high peptide occupancy determined serologically (Fig. 2).

Effect of the Y84A Mutation on Peptide Displacement—We envisioned two models consistent with the stable nature and pronounced peptide occupancy of the SCT. Either the spacer somehow strengthens the interaction of the peptide with the H-chain, or the covalent linkage permits the peptide to rapidly rebound after dissociation such that the trimeric structure is maintained. To shed light on this issue, we compared the displacement of the OVA peptide from native OVA-Kb complexes versus the SCT molecules. Cells expressing Kb (loaded with exogenous OVA), the OVA-Kb SCT, or the OVA-Kb Y84A SCT were incubated with various concentrations of a competing Kb-binding peptide (SIYRYYGL). The SIYR peptide is a known Kb ligand of comparable affinity to the OVA peptide (33). After overnight incubation, displacement of the OVA peptide by the SIYR peptide was revealed as a decrease in mAb 25-D1.16 staining intensity. Fig. 4 demonstrates that the OVA peptide linked to either the SCT or the Y84A SCT was displaceable using high concentrations of the competing peptide. By contrast, native OVA-Kb complexes were unaffected under these conditions, even at 500 μM SIYR peptide. This implied that the cell-surface stability and high level of peptide occupancy of the SCTs were conferred by their ability to efficiently rebind the attached peptide. Interestingly, the SCT with the Y84A mutation was ~5 times more refractory to peptide displacement. This effect could result from decreased binding of competing
peptides to the mutant SCT and/or fortuitous stabilizing interactions with the covalent linker that lead to improved association of the tethered peptide, a phenomenon that has been suggested for class II molecules with covalent peptides (36).

**CTL Detection of SCTs with and without Y84A**—We previously showed that targets expressing the OVA-Kb SCT are recognized by cognate CTL (OT-1) to a similar degree as peptide-fed native Kb targets (21). Although Y84A SCT molecules are recognized very well by mAb 25-D1.16, it was important to determine whether this mutation alters CTL detection. As shown in Fig. 5, OT-1 CTL efficiently recognized target cells expressing the OVA-Kb Y84A molecules. Indeed, lytic activity against these targets was comparable to that against peptide-fed targets and somewhat better than that against SCT without the Y84A mutation. Improved CTL detection of the SCTs with the Y84A mutation is consistent with their improved detection using mAb 25-D1.16. Furthermore, these observations indicate that mutation of Tyr24 does not affect recognition of OVA-Kb by OT-1 T cells. It is noteworthy that this residue can also be mutated in HLA A2 without loss of CTL detection (42).

**Characterization of Recombinant OVA-Kb SCTs**—Because our data revealed that SCTs are quite stable at the cell surface and make excellent CTL targets, we thought that recombinant SCTs might be useful for a variety of applications. To this end, versions of the OVA-Kb SCTs lacking the leader sequence (replaced by a Met for initiation) and the transmembrane domain were expressed in E. coli. Recombinant material from purified inclusion bodies exhibited the expected molecular mass upon SDS-PAGE, and N-terminal sequence analysis revealed that the initiator Met was efficiently removed by the bacteria, exposing the desired N terminus (Ser) (data not shown). This material was subjected to refolding under standard class I refolding conditions. Soluble refolded material was obtained with efficiency comparable to what we routinely observed for conventional class I refolding.

Recombinant SCT molecules were prepared for OVA-Kb and OVA-Kb Y84A and were initially compared using ELISA. Equivalent amounts of both SCTs were bound to ELISA plates and then probed with various mAbs. Fig. 6 gives the results from a representative experiment demonstrating that the recombinant molecules reacted strongly with each mAb tested, including mAb 25-D1.16. Importantly, when comparing the SCT and Y84A SCT, the ratio of the signals obtained using mAb 25D-1.16 versus B8-24-3 was remarkably similar to what we observed for the corresponding molecules expressed in mammalian cells (compare with Fig. 2 and Table I). In other words, the OVA-Kb signal (mAb 25-D1.16) was improved relative to the total folded K\(^\text{b}\) signal (mAb B8-24-3) for the Y84A SCT molecules. This finding supports the model that the improved mAb 25-D1.16 recognition of the Y84A SCT mutant is due to better tolerance of the linker. Indeed, one explanation mentioned above for the suboptimal mAb 25-D1.16 recognition of the wild-type SCT expressed in mammalian cells could be that some endogenous peptides are bound in place of the covalent peptide, thus reducing the mAb 25-D1.16 staining. This is clearly not the case because the recombinant molecules showed the same pattern of suboptimal mAb 25-D1.16 staining that was improved with the Y84A mutation, yet these molecules were refolded in the absence of any exogenous peptides.
Production of Tetramers Using SCTs—The ELISA data obtained using the recombinant SCTs indicated that they assumed the correct conformation. Therefore, we were interested in determining whether SCTs could be used to generate tetramers for enumerating antigen-specific T cells. Given the stability of the OVA-Kb SCT at the cell surface, we reasoned that tetramers made with SCTs might prove advantageous under certain circumstances. To test the feasibility of using SCTs for tetramers, we produced recombinant OVA-Kb Y84A molecules that included a C-terminal biotinylation target sequence. After enzymatic biotinylation of refolded molecules, tetramers were generated by the addition of phycoerythrin-labeled streptavidin. The tetramers were tested by staining of splenocytes from OT-1 transgenic mice (positive control) or C57BL/6 mice (negative control) and compared with staining using native OVA-Kb tetramers prepared using conventional methods. Analysis of cells stained for CD8 versus the tetramers is shown in Fig. 7. The SCT-based tetramers were clearly functional, as they readily stained CD8+ cells from the OT-1 mice, but not from naive B6 animals, similar to the conventional tetramers. Thus, SCTs can be used to generate tetramers, and the unique properties of SCTs may offer improvements over conventional tetramers. In support of this conclusion, Greten et al. (20) recently reported the construction of bivalent staining reagents consisting of human HLA A2 molecules with covalently linked peptide and β2m.

DISCUSSION

In this study, we probed the immunological presentation of SCTs and explored salient mechanisms explaining their stability. Given the importance of peptide occupancy for the integrity of class I molecules, we hypothesized that the remarkable stability of SCT is due to either (i) their ability to bind peptide tighter than normal class I molecules, perhaps conferred by novel linker/heavy chain interactions, or (ii) their ability to efficiently “rebind” the attached peptide after dissociation occurs. The data presented here are most consistent with the latter model. Indeed, the peptide displacement data indicate that the OVA peptide is bound more weakly by the SCT compared with native OVA-Kb complexes. In retrospect, this was not wholly unexpected because many of the conserved interactions of the H-chain with the C terminus of the peptide have been abolished in the SCT due to the presence of the linker (9, 10). Nonetheless, the net effect of having the peptide covalently linked in the SCT more than compensates for the loss of these peptide/H-chain interactions, such that OVA-Kb SCTs remain intact, have a prolonged cell-surface half-life, and exhibit a high level of steady-state peptide occupancy (Ref. 21 and this work).

The continuous re-binding of peptide does not appear to affect CTL or antibody detection of the OVA-Kb SCT. Furthermore, it is unlikely that peptide displacement would be a significant problem in vivo because high concentrations of competing peptides of the required length and sequence are unlikely to be encountered under normal conditions. Certainly, the presence of naturally processed peptides in the ER of cells expressing SCTs is insufficient to result in significant displacement of the covalent peptide in the SCT. Rather, we have found that cells expressing OVA-Kb SCTs are potent stimulators of CTL both in vitro and in vivo (21) (data not shown). Furthermore, we have now made SCTs of several different mouse and human H-chains bound by various of their known respective ligands, and all SCTs tested thus far are superior targets for CTL (data not shown). It is less clear, however, whether incorporation of the Y84A mutation into the SCT will improve T cell interaction for all peptide/class I complexes. As noted above, mAb 25-D1.16 binds the OVA-Kb complex toward the C-terminal end of the peptide (35) and thus would be expected to be influenced by the linker more than T cells. Although we have observed a trend toward somewhat better CTL recognition of the OVA-Kb Y84A molecule (Fig. 6) (data not shown), our preliminary analysis of the HLA A2 SCT suggests that there is little difference in T cell recognition afforded by the Y84A mutation with this particular H-chain (data not shown). Thus, the Y84A mutation has provided important mechanistic insights into the nature of peptide binding by the SCT, but may not be a universal requirement for making SCTs with optimal T cell recognition.

SCTs could have several applications in basic research as well as in the diagnosis and treatment of human diseases. As reported here, SCTs can be used to make tetramers, which are increasingly being used to monitor the dynamics of antigen-specific CD8+ T cell responses (43). Two limitations of tetramers technology are (i) their dependence on high affinity peptides.
to initially fold sufficient quantities of class I H-chains and (ii) problems of peptide dissociation rendering assembled tetramers unstable. Indeed, several well characterized antigenic peptides detected by CDS\(^{+}\) T cells have relatively weak binding to class I molecules (e.g. mouse L\(^{1}\) ligands p2Ca and tum\(^{-}\)) (44–46). Perhaps using SCT approaches will help in the construction and stability of tetramers made with lower affinity peptides. Furthermore, tetramers are more recently being used to modulate CDS\(^{+}\) T cells in vitro to study pathways of CDS\(^{+}\) T cell activation (47). Again, tetramers with covalently attached peptides may be more stable and thus more potent immunomodulators.

Probably the most exciting potential application of SCT technology will be their use in DNA vaccination protocols. We have found that SCTs can stimulate antigen-specific CDS\(^{+}\) T cell and antibody responses following DNA vaccination (21) (data not shown). Although SCT potency in antibody and CTL stimulation may reflect SCT surface stability, additional factors warrant consideration. For example, even though SCTs remain intact at the cell surface, they do have the potential to multimerize by sharing subunits. Such multimers (probably dimers) may provide a structure that enhances antigen presentation by display of a more defined topology relative to monomeric forms. In any case, in vitro expression of SCTs following DNA vaccination not only stimulates antigen-specific T cell responses, but should also be resistant to virus-encoded immune evasion proteins that shut off peptide supply, such as ICP47 of herpes simplex virus (48–50) and US6 of human cytomegalovirus (51).

In addition, the pre-assembled nature of SCTs and/or their rapid assembly kinetics may render them more resistant to virus-encoded immune evasion proteins (e.g. herpesvirus 68 protein mK3) (52). Perhaps using SCT approaches will help in the construction of highly stable forms of antigen presentation not only stimulates antigen-specific T cell responses, but should also be resistant to virus-encoded immune evasion proteins that shut off peptide supply, such as ICP47 of herpes simplex virus (48–50) and US6 of human cytomegalovirus (51).

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Enhanced Immune Presentation of a Single-chain Major Histocompatibility Complex Class I Molecule Engineered to Optimize Linkage of a C-terminally Extended Peptide

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