Cutting Edge: Single-Chain Trimers of MHC Class I Molecules Form Stable Structures That Potently Stimulate Antigen-Specific T Cells and B Cells

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We report in this work the expression and characterization of class I molecules expressed as single-chain trimers consisting of an antigenic peptide-spacer-β2-microglobulin-spacer H chain. Our results indicate that these single-chain constructs assemble efficiently, maintain their covalent structure, and are unusually stable at the cell surface. Consequently, these constructs are at least 1000-fold less accessible to exogenous peptide than class I molecules loaded with endogenous peptides, and they are potent simulators of peptide-specific CTL and Abs. Our combined findings suggest that single-chain trimers may have applications as DNA vaccines against virus infection or tumors. The Journal of Immunology, 2002, 168: 3145–3149.

Class I H chains require full assembly with β2-microglobulin (β2m)3 and peptide to be stably expressed at the cell surface at levels sufficient to induce optimal T cell immunity (1–4). Class I molecules that fail to complete assembly accumulate in the endoplasmic reticulum (ER) and are targeted for degradation (5). There have been two seminal findings regarding class I assembly in recent years. First, assembly in the ER involves complex interactions with ER proteins that facilitate full class I assembly (6). Second, pathogens and tumors evolve diverse mechanisms to block class I assembly as a means of evading immune detection (7, 8).

As a novel approach to make class I molecules less dependent on chaperone assistance and more resistant to down-regulating mechanisms of viruses, we have produced single-chain class I molecules. There have been two reports of successfully engineering class I molecules by directly linking a peptide ligand to the H chain via a short linker (9, 10), an approach not applicable to most class I/peptide complexes (our unpublished data). Others have reported coupling β2m to the N terminus of class I molecules with a spacer (11–14), or covalent linkage of the specific peptide to free β2m which can then associate with H chains to generate functional trimers (15–17). Nonetheless, it remains unclear the extent to which the covalent attachment of peptides to β2m excludes the binding of competing free peptide ligands. We report in this work the first demonstration of linking all three components of class I as a single-chain trimer (SCT), using an approach applicable to all mouse and human class I/peptide complexes thus far. Furthermore these SCT efficiently exclude competing peptides and are potent stimulators of Ag-specific T and B cells.

Materials and Methods

Mice

B6 (H-2b), BALB/c (H-2d), and (C3H × B6)F1 (H-2kx) mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). OT-1 transgenic mice (18) were kindly provided by R. Lorenz (Washington University School of Medicine, St. Louis, MO).

Cell lines, Abs, and peptides

L cell line LMI.18 (H-2d) (19) was a gift from P. Kourilsky (Institut National de la Santé et de la Recherche Médicale, Institut Pasteur, Paris, France); 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 (20); B8–24–3 (American Type Culture Collection, Manassas, VA), which recognizes folded Kβ; and 25D–1.16, which recognizes Kβ + SIINFEKL peptide (21). The OVA-derived peptide (SIINFEKL) (22) and SIYR (SIYRYYGL) (23) were synthesized on a peptide synthesizer (model 432A; PE Applied Biosystems, Foster City, CA).

DNA constructs

Constructs were generated using standard techniques and were confirmed by DNA sequence analysis. The β2mKβ constructs encode β2m followed by a linker of 15 or 20 residues consisting of (G6S)5–4 followed immediately by the mature Kβ H chain sequence. The OVAβ2mKβ constructs encode the leader sequence of β2m followed immediately by the SIINFEKL sequence, then a linker of 10 or 15 residues (G6S)5–4. This first linker is followed by the mature β2m sequence, the second linker of 15–20 residues, (G6S)5–4, then the mature Kβ sequence. Constructs were expressed from the pRESneo vector (Clontech Laboratories, Palo Alto, CA). The 64–3–7 epitope-tagged Kβ mutant (KβR48Q,R50P) was described previously (24).

Cytotoxic T lymphocytes

The L1−alloreactive CTL clone, 2C (a gift from H. Eisen, Massachusetts Institute of Technology, Cambridge, MA), was maintained by weekly restimulation with irradiated BALB/c splenocytes as previously described (25). When freshly explanted splenocytes were used, 7.5 × 105 cells were cultured with 3.5 × 106 irradiated splenocytes (2000 rad) for 1 wk in the absence of IL-2 or 3.5 × 105 irradiated L cells (10,000 rad) weekly for 2 wk in the absence of IL-2. Thereafter, 2.5–5 × 105 cells were restimulated weekly with 5 × 106 irradiated splenocytes or 3.5 × 105 irradiated L cells

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3 Abbreviations used in this paper: β2m, β2-microglobulin; ER, endoplasmic reticulum; SCT, single-chain trimer.

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in the presence of 10 U/ml rIL-2. When peptide was included the concentration was $10^{-4}$ M in the absence of IL-2 and $10^{-3}$ M in the presence of IL-2.

Flow cytometry

Viable cells, gated by forward and side scatter, were analyzed on a FACS-Calibur (BD Biosciences, San Jose, CA) and data were analyzed using CellQuest software (BD Biosciences). Assays for peptide incubation and turnover of class I were performed as previously reported (24).

Biochemistry

Immunoprecipitations and blots were performed as described (26). Proteins were visualized using ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

DNA vaccination

DNA was prepared and immunizations were conducted according to Corr et al. (27). Briefly, BALB/c mice were injected i.m. in the quadriceps with 100 μg of DNA encoding OVA/β2m/Kb. Injections were given twice, separated by a 2-wk interval. Four weeks later mice were bled and tested for Ab production by flow cytometry.

Results and Discussion

Experimental approach

In this study, several class I SCT constructs were produced. Although they vary in content, each of these SCT consists of the following elements beginning with the amino terminus: the leader sequence of β2m, the peptide comprising a ligand for the H chain, the first flexible linker of 10 or 15 residues, the mature portion of murine β2m, the second flexible linker of 15 or 20 residues, and, finally, the mature portion of the H chain. To serve as controls, constructs were also made with only β2m covalently attached to the H chain. These constructs consist of the entire coding region of β2m linked via a 15- or 20-residue spacer to the mature portion of the respective H chain.

Surface expression and functional capabilities of SCTs

Single-chain constructs were initially generated with mouse class I L4 molecules and several different ligands. These SCT constructs resulted in high levels of surface expression and immune recognition by mAb and CTL to L4 (data not shown). However, our characterization of Kb SCTs with SIINFEKL peptide (OVA) and β2m is the most comprehensive due to well defined OT-1 T cells and mAb 25D-1.16 that specifically recognizes Kb/OVA complexes (18, 21).

To test whether spacer length contributed the immune detection of SCT, a set of OVA/β2m/Kb constructs was produced with linkers of different lengths. In addition to varying the linker length, the epitope detected by mAb 64-3-7 was introduced into the Kb H chain to allow detection of peptide empty forms as previously reported (24, 26, 29). As shown in Fig. 1, all of the constructs gave rise to high levels of expression of folded Kb molecules (B8-24-3+). However, when examined for the presence of open conformers (64-3-7-”), only the Kb (Fig. 1A) and β2m (L20) Kb (Fig. 1B) constructs expressed an appreciable amount, which disappeared with addition of exogenous peptide (data not shown), thus reaffirming their “peptide-empty” nature. In stark contrast, the SCT constructs expressed barely detectable levels of open conformers. Thus, the covalent OVA peptide must be able to occupy the Kb peptide binding groove virtually all the time. Remarkably, the linker combination of (15/20) provided significantly better mAb 25D-1.16 reactivity than the other two combinations. In parallel with the FACS profiles in Fig. 1A, the recognition by OT-1-derived T cells was also the best for these transfectants (Fig. 1B).

Thus, increasing the length of the flexible linkers results in improved recognition of the OVA/β2m/Kb construct by both the mAb 25D-1.16 and OT-1 T cells. This improved recognition with longer spacers in SCT could reflect better peptide positioning and/or reduced steric hindrance for TCR and Ab interactions.

Conceptually, it is intriguing how SCT constructs maintain their structural integrity, because the class I ligand binding groove is closed where the C terminus of the peptide binds, and this architecture contributes significantly to strong peptide binding. Immune recognition of SCT indicates that they must orient peptide in a manner very similar to that of noncovalently attached peptides. Therefore, it seems likely that the linker residues protrude out from the C terminus of the peptide binding groove. Indeed, a structural model explaining how SCT bind peptide can be extrapolated from studies of Collins et al. (30). They reported a structure of a decamer peptide that bound with its C-terminal residue bound outside the HLA-A2 peptide binding site. To allow the peptide to extend beyond the binding groove and maintain tight binding, the conserved H chain residue Lys146 was found to rotate to form new bonds with the protruding C-terminal peptide residue. In further support of a protrusion theory, our preliminary data suggest that the already significant mAb 25D-1.16 recognition of OVA/β2m/Kb (15/20) construct can be improved further by introducing point mutations predicted to open the peptide binding groove at the C terminus (L. Lybarger, Y. Yu, J. Connolly, and T. Hansen, manuscript in preparation).

SCT are at least 1000-fold less accessible to loading with exogenous peptide

To assess the stability of the covalent peptide that is anchored in the peptide binding groove, peptide competition assays were performed. To do this, the 2C CTL clone was used because it can
recognize K\textsuperscript{b} in complex with the SIYR peptide (23), which has an affinity for K\textsuperscript{b} that is comparable to the OVA peptide (31). When β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} or OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} transfectants were compared as targets for 2C T cells after overnight incubation with graded doses of SIYR peptide (Fig. 2), the OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} construct was remarkably resistant to displacement by exogenous SIYR peptide at a concentration as high as 10\textsuperscript{-7} M. Contrary to this, there was significant lysis of β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} transfectants at a concentration as low as 10\textsuperscript{-10} M. This finding suggests that the OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} construct is >1000-fold less accessible to loading by an exogenous peptide of comparable affinity, when compared with the β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} constructs loaded with endogenous peptides. Thus, the covalent peptide is stably bound in the SCT peptide binding groove.

Biochemical integrity of the SCT molecules

The finding that the peptide-binding groove of the OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} molecule was relatively inaccessible to exogenous peptide suggested that all of the components of the SCT remain intact at the cell surface (Fig. 2). To examine the integrity of these molecules further, biochemical analyses were performed to compare K\textsuperscript{b}, β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b}, and OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b}. Each of these molecules was immunoprecipitated from respective L cell transfectants and immunoblotted to compare the relative m.w. of all three K\textsuperscript{b} constructs. As shown in Fig. 3A, mAb 64-3-7 (specific for open H chains) precipitated high levels of K\textsuperscript{b} but low to undetectable amounts of β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} and OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b}. By contrast, B8-24-3 (specific for folded K\textsuperscript{b}) was able to precipitate significant amounts of all three constructs. The differential reactivity with these two mAbs demonstrates that the covalent attachments to K\textsuperscript{b} reduced the levels of open conformers existing at steady-state. In addition, this experiment clearly demonstrated that the β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} and OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} covalent constructs exhibit a slower migration consistent with their rapid ER turnover when compared with K\textsuperscript{b} alone as determined in pulse-chase experiments (data not shown).

To demonstrate that the OVA peptide was not undergoing proteolysis from the SCT and then rebinding as an unattached peptide, precipitates were performed using mAb 25-D1.16. Fig. 3B demonstrates that mAb 25-D1.16 precipitated OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} as well as β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} and K\textsuperscript{b} molecules (only after the latter two constructs were incubated overnight with exogenous OVA peptide). Importantly, the SCT migrated slightly more slowly than the β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} construct that was precipitated from cells incubated with exogenous peptide (Fig. 3B, indicated by an arrow). Thus, this experiment demonstrates that OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} molecules retain covalently attached OVA peptide rather than rebinding free OVA peptide after proteolysis of the SCT.

As additional evidence for the integrity of SCT molecules, their surface turnover was compared with that of noncovalently attached K\textsuperscript{b} molecules with and without overnight incubation with OVA peptide. As shown in Fig. 3C, K\textsuperscript{b} molecules loaded with endogenous peptides have a half-life of ~8 h, whereas K\textsuperscript{b} molecules loaded with noncovalently bound OVA peptide have a half life of ~16 h. Remarkably, the OVA SCT were more stable than the K\textsuperscript{b} loaded with detached OVA, with no detectable turnover within the 16 h tested. Thus, the integrity of SCT molecules is supported by their 1) resistance to peptide exchange, 2) biochemical detection, and 3) stability at the cell surface.

SCT are immunogenic

The data presented above evince that SCT can assume structures that are recognized by Abs and T cells that were raised originally against native peptide/class I complexes. We next examined the extent to which SCT were capable of stimulating immune responses that would be specific for the native peptide/class I structures. To test the ability of the single-chain class I molecules to generate a T cell response, we compared the ability of LM1.8 (H\textsuperscript{2})\textsuperscript{b} cells expressing OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} vs β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} fed exogenous OVA peptide (10\textsuperscript{-4} M) to induce K\textsuperscript{b}/OVA specific T cells in vitro. For this experiment, responder T cells from (C3H(H-2\textsuperscript{k}) × B6(H-2\textsuperscript{k})) F1 were used as targets for 2C T cells after overnight incubation with exogenous SIYR peptide before lysis. Precipitates were digested or mock-digested with Endo H before immunoblot with mAb 64-3-7. C. Surface decay following brefeldin A addition (5 μg/ml) was monitored on LM1.8 cells expressing K\textsuperscript{b} (circles), K\textsuperscript{b} previously loaded with exogenous OVA peptide (diamonds), or OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} (15/20) (squares). Cells were stained with mAb B8-24-3 (open symbols) and mAb 25-D1.16 (filled symbols). Mean fluorescence values were obtained at each time point and plotted as a percentage of the starting signal.

FIGURE 2. Resistance of the OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} construct to displacement by high-affinity K\textsuperscript{b} binding peptide. LM1.8-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} cells (filled symbols) or LM1.8-OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} cells (open symbols) were tested as targets for recognition by 2C T cells. The targets were incubated overnight with the indicated molar concentration of peptide.

FIGURE 3. Biochemical comparisons of the K\textsuperscript{b} constructs. For all experiments, the following cells were used: LM1.8-K\textsuperscript{b}, LM1.8-β\textsubscript{m}(L20)K\textsuperscript{b}, and LM1.8-OVA-β\textsubscript{m}(L20)K\textsuperscript{b} (15/20). The K\textsuperscript{b} constructs used were tagged with the 64-3-7 epitope. A. Lysates from equivalent numbers of the indicated cells were precipitated (i.p.) with the mAb listed across the top. Immunoblot of the precipitates, using mAb 64-3-7, is shown. B. Lysates from the indicated cells were immunoprecipitated with mAb 25-D1.16. In some cases, cells were incubated overnight with exogenous OVA peptide before lysis. Precipitates were digested or mock-digested with Endo H before immunoblot with mAb 64-3-7. C. Surface decay following brefeldin A addition (5 μg/ml) was monitored on LM1.8 cells expressing K\textsuperscript{b} (circles), K\textsuperscript{b} previously loaded with exogenous OVA peptide (diamonds), or OVA-β\textsubscript{m}\textsuperscript{b-K}\textsuperscript{b} (15/20) (squares). Cells were stained with mAb B8-24-3 (open symbols) and mAb 25-D1.16 (filled symbols). Mean fluorescence values were obtained at each time point and plotted as a percentage of the starting signal.
2β2m, mice were used that should respond only to Kb/OVA complexes presented by either OVA-βm,mKb or peptide-fed βm,mKb. Successful generation of Ag-specific CD8+ T cells typically requires in vivo priming, intracellular peptide loading, or Ag-pulsed, purified dendritic cells (22, 32). However, high levels of Kb/OVA-species lysis were observed after five weekly rounds of in vitro stimulation with the OVA-βm,mKb construct (Fig. 4A). By comparison, after five weekly rounds of stimulation with cells expressing the βm,mKb construct and fed 10−8 M continuous OVA peptide, little if any Kb/OVA-specific lysis was observed (Fig. 4A).

The high level of occupancy with a single peptide is likely a contributing factor to the strong immunogenicity of SCT as evidenced by their ability to generate peptide-specific T cells after in vitro culture. Another contributing factor could be an impaired interaction with NK inhibitory receptors on NK cells or T cells. Indeed, Chung et al. (14, 33) demonstrated that a linker extending from the C terminus of β2m to the N terminus of the H chain blocks NK receptor interaction. Likewise, it is also worth considering that SCT could have impaired interaction with CD8 coreceptors. If such were true, T cells resulting from repeated immunization with SCT could be relatively CD8 independent and thus have a higher such were true, T cells resulting from repeated immunization with DNA encoding OVA.

Remarkably, these Abs were found to be only two injections of DNA, two of six BALB/c recipients made significant anti-Kb Abs. Together these findings demonstrate that the OVA-βm,mKb single-chain construct is highly immunogenic due to its capacity to remain covalently attached and to stimulate the generation of peptide-specific, class I-restricted, CD8+ T cells and Abs.

Collectively, these studies demonstrate that SCT appear to fold correctly when assessed by mAb and T cell recognition. Furthermore, biochemical analyses and peptide competition experiments demonstrate that SCT remain intact, and functional analyses indicate that SCT perform as well as, or in some cases better than, their noncovalent counterparts. We have applied this technology to two murine class I molecules using multiple ligands. In addition, we have evidence that this same approach can be used to generate functional HLA class I SCT (L. Lybarger, Y. Yu, J. Connolly, and T. Hansen, manuscript in preparation). These properties make SCT molecules highly attractive for applications as DNA vaccines or probes of mechanisms of class I assembly in normal and pathogenic conditions.

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FIGURE 4. A. Superior immunogenicity of OVA-βm,mKb (15/20) stimulators over peptide-fed βm,mKb (20/20) stimulators. Lysis of RMA targets in the absence (open symbols) or continuous presence (filled symbols) of 1 μM OVA peptide by (C3H × B6)F1 effectors after five weekly stimulations with LM1.8-OVA-βm,metKb (15/20) cells (triangles) or LM1.8-βm,mL20-yeKb cells fed continuous OVA peptide (circles). B, Vaccination of BALB/c mice with DNA encoding OVA-βm,mKb elicits Abs specific for Kb/OVA complexes. Four weeks after the second DNA injection, sera were obtained and tested for reactivity against LM1.8-Kb cells, with or without overnight incubation in the presence of the Kb-binding peptides, OVA, or VSV8. Serum from mouse 3 is shown, as is staining of the same cells with mAb B8-24-3 for comparison.


