Functional cell surface expression by a recombinant single-chain class I major histocompatibility complex molecule with acis-active β2-microglobulin domain

ARTICLE *in* EUROPEAN JOURNAL OF IMMUNOLOGY · NOVEMBER 1994

Impact Factor: 4.03 · DOI: 10.1002/eji.1830241110 · Source: PubMed

6 AUTHORS, INCLUDING:

Steven Kozlowski
U.S. Food and Drug Administration
47 PUBLICATIONS **1,804** CITATIONS
SEE PROFILE

David H Margulies
National Institute of Allergy and Infectious…
180 PUBLICATIONS **8,398** CITATIONS
SEE PROFILE

Michael G Mage
U.S. Department of Health and Human Ser…
74 PUBLICATIONS **2,279** CITATIONS
SEE PROFILE

Li Lee●,
Louise McHugh●,
Randall K. Ribaudo▽■,
Steven Kozlowski▲■,
David H. Margulies■ and
Michael G. Mage●

Laboratory of Biochemistry, National Cancer Institute● and Laboratory of Immunology, National Institute of Allergy and Infectious Diseases■, National Institutes of Health, Bethesda

Functional cell surface expression by a recombinant single-chain class I major histocompatibility complex molecule with a *cis*-active β_2-microglobulin domain

As a preliminary step towards the use of cell surface single-chain class I major histocompatibility complex (MHC) molecules as T cell immunogens, we have engineered a recombinant gene encoding a full-length cell surface single-chain version of the H-2D^d class I MHC molecule (SCβD^{d}m) which has β_2-microglobulin (β_2m) covalently linked to the amino terminus of a full-length H-2D^d heavy chain via a peptide spacer. The single-chain protein is correctly folded and stably expressed on the surface of transfected L cells. It can present an antigenic peptide to an H-2D^d-restricted antigen-specific T cell hybridoma. When expressed in peptide-transport-deficient cells, SCβD^{d}m can be stabilized and pulsed for antigen presentation by incubation with extracellular peptide at 27° or 37 °C, allowing the preparation of cells with single-chain molecules that are loaded with a single chosen antigenic peptide. SCβD^{d}m can be stably expressed in β_2m-negative cells, showing that the single-chain molecule uses its own β_2m domain to achieve correct folding and surface expression. Furthermore, the β_2m domain of SCβD^{d}m, unlike transfected free β_2m, does not rescue surface expression of endogenous class I MHC in the β_2m-negative cells. This strict *cis* activity of the β_2m domain of SCβD^{d}m makes possible the investigation of class I MHC function in cells, and potentially in animals, that express but a single type of class I MHC molecule.

1 Introduction

Class I MHC molecules are found on the surface of nearly all vertebrate cells. This heterodimer of a 46-kDa heavy chain with the 12-kDa light chain β_2-microglobulin (β_2m), can present peptides derived from intracellular pathogens to CD8$^+$ cytotoxic T lymphocytes [1–4]. Current evidence suggests that the β_2m chain plays an important role in MHC class I intracellular transport [5, 6], peptide binding [7–13], and conformational stability [6, 7, 14–17]. For most class I molecules, a heterotrimer consisting of the MHC class I heavy chain, self- or antigenic peptide and β_2m, is required for maturation and stable cell surface expression [11–12, 18].

Recently, we and others have explored the expression [19–22] and function in antigen presentation [19] of soluble single-chain MHC class I molecules. These are genetically engineered proteins that covalently link both the heavy and light chain components via an appropriate peptide spacer and lack the naturally occurring transmembrane portion of the molecule. Thus, the natural three-component MHC structure (peptide, light chain, and heavy chain) is reduced to two, limiting the structural degrees of freedom. The soluble single-chain molecules fold correctly, as assessed by their reactivity with conformation-sensitive antibodies. Their peptide binding specificity, analyzed in detail in the case of single-chain K^d [21] and K^b [22] is the same as that of the wild-type two-chain molecules. This suggests that they may provide an appropriate vehicle for delivery of antigenic peptides in immunization. The soluble single-chain H-2D^d molecule that we have described [19], which has β_2m at the amino terminus, was able to stimulate an antigen-sensitive H-2D^d-restricted T cell hybridoma. Although it is valuable to study the class I molecule as a soluble purified component, its natural function in immune responses is performed as a cell surface molecule. The soluble single-chain D^d, unlike the soluble K^d and K^b single-chain molecules [20–22], has the α3 domain at the carboxyl terminus, offering the possibility of its extension to include a natural transmembrane region and cytoplasmic domain that would permit cell surface expression. We describe here in detail the construction, expression, and function of such a membrane-bound single-chain H-2D^d molecule, SCβD^{d}m, that has a transmembrane and cytoplasmic domain. Because it provides the two protein subunits in a *cis* form, SCβD^{d}m is effectively expressed at the cell surface, even in cells defective for the expression of β_2m.

[I 13117]

▽ Current address: Carcinogenesis and Development Program, Lab. of Immune Cell Biology, NCI, Bg. 10, Rm. 1B42, NIH, Bethesda MD 20892, USA.

▲ Current address: Center for Biologics Evaluation and Research, Food and Drug Administration, Bg 29, Rm. 330, Bethesda MD 20892, USA.

Correspondence: Michael G. Mage, Laboratory of Biochemistry, NCI, Bg. 37, Rm. 4C28, NIH, Bethesda, MD 20892, USA

Abbreviations: **β_2m:** β2-Microglobulin

Key words: Major histocompatibility complex class I / Single chain / β2-microglobulin

0014-2980/94/1111-2633$10.00+.25/0

2 Materials and methods

2.1 Antibodies

The following class I monoclonal antibodies were used: 34-5-8 (anti-H-2D^d α1α2) [23]; 34-2-12 (anti-H-2D^d α3) [24]; 28-14-8 (anti-H-2L^d, D^b α3) [25]; 11-4-1 (anti-H-2K^k) [26]; W6/32 (anti-HLA-A, B, C) [27] and BB7.2 (anti-HLA/A2) [27], were from the American Type Culture Collection. Anti-human β_2m BM63 was obtained from Sigma Chemical Co.

2.2 Plasmid construction

The structure of the recombinant gene encoding the membrane single-chain class I sequence is shown schematically in Fig. 1. The technique used to obtain chimeric soluble single-chain class I sequences has been described in detail [19]. The β_2m-encoding cDNA and full-length heavy chain H-2D^d [7, 19] were amplified by PCR from cDNA clones respectively. The four primers which were used in PCR are 3667 (CCA GGC GCT GAC ATG GCT CGC TCG GTG ACC CTG), containing the sequence for a Sal I site immediately 5′ of the signal peptide sequence, 3552, a 3′ antisense primer (GCC GCC ACC CGA GCC GCC TCC GCC GCT ACC GTC ACC TCC CAT GTC TCG ATC CCA GTA), containing the sequence for the 3′ end of β_2m and a portion of a 20-amino acid spacer, 3533 (GGC GGC TCG GGT GGC GGC GGA AGC GGC GGA GGT GGA TCC GGC TCA CAC TCG CTG AGG), containing a portion of the peptide spacer and the 5′ end of the H-2D^d heavy chain and 3674 (CCA GGC AAG CTT CAA GTC CAC ACT AGG CAG CT), a 3′ antisense primer containing the H-2D^d 3′ cytoplasmic region sequence and a Hind III site. The two PCR products were mixed and spliced by overlap extension [28] in order to generate a 1.4-kb DNA fragment encoding β_2m linked via a 20-amino acid peptide spacer to the amino terminus of a full-length H-2D^d heavy chain. The recombinant gene was sequenced using SEQUENASE Version 2.0 (United States Biochemical). Two point mutations, one that was present in the original H-2D^d cDNA, and one in the spacer that was inadvertently encoded in primer 3552 were identified and corrected by *in vitro* mutagenesis using the "Altered Sites" kit from Promega. The single-chain construct was cut with Sal I and Hind III, ligated into the eukaryotic expression vector pHβAPr-1 neo [29], under control of the human β-actin promoter by standard methods to yield the recombinant plasmid, pSCβD^{d}m.

2.3 Cell cultures and transfection

The cell lines used in this study are listed in Table 1. They were maintained at 37 °C, 6.5% CO_2 in complete DMEM containing 10% FCS, 2 mM glutamine, 1% non-essential amino acids, 50 μM 2-mercaptoethanol, and 50 μg per ml gentamycin. Cells were transfected with the recombinant plasmid pSCβD^{d}m by calcium phosphate precipitation. Clones resistant to G418 were screened for expression of D^d epitopes by flow cytometry. The β_2m-deficient human kidney carcinoma cell line KJ29 [30] was a generous gift from Dr. Roberto Gambari (Centro Interdipartimentale di Biotecnologia, Universita' di Ferrara, Italy). The human β_2m-transfected clone, KJ29.β_2m, a gift from Dr. Patricio Giacomini (Regina Elena Cancer Institute, Rome, Italy) and Dr. Michele Fiscella (Laboratory of Cell Biology, National Cancer Institute), was prepared by transfecting KJ29 cells with a vector containing human β_2m cDNA (P. G. and M. F., unpublished data).

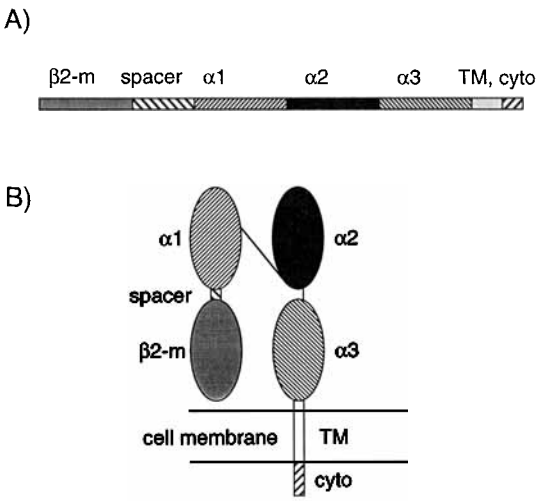

Figure 1. Full-length single-chain H-2D^d. (A) cDNA encoding a peptide spacer between β_2m and D^d heavy chain. (B) Full-length single-chain protein, SCβD^{d}m, with the β_2m domain at the amino terminus.

Table 1. Cells used in this study

Cell	Description	Reference
DAP3	Thymidine kinase-negative L cell, H-2^k	[43]
EE2H3	From mid-somite stage C3H embryo, class I-negative	[44]
KJ29	Human kidney carcinoma, β_2m-negative	[30]
KJ29.β_2m	KJ29 transfected with human β_2m (clone 19)	This study
SKT4.5	DAP3 transfected with H-2D^d	[38]
LKD8	EE2H3 transfected with H-2D^d	[16]
DAP3.MSC	DAP3 transfected with single-chain full-length H-2D^d	This study
EE2H3.MSC	EE2H3 transfected with single-chain full-length H-2D^d	This study
KJ29.MSC	KJ29 transfected with single-chain full-length H-2D^d	This study
B4.2.3	T hybridoma, H-2D^d + peptide p18I10-restricted	[9]

2.4 Immunofluorescence and flow cytometry

Cells were reacted with mAb 34-5-8, 34-2-12, 11-4-1, W6/32, BB7.2 or BM-63 followed by FITC-conjugated goat F(ab')$_2$ anti-mouse Ig (Zymed Laboratories, Inc.). Dead cells were excluded from the analysis by staining with propidium iodide (0.5 μg/ml). Cells were analyzed using a FACS Analyzer or FACScan (Becton Dickinson).

2.5 T cell stimulation assay

Transfected and untransfected control cells (5×10^5 cells/well) were placed in 96-well polystyrene tissue culture plates (Costar) at 37 °C overnight. After washing with PBS, cells were incubated at 37 °C for 3 h, with the H-2D^d-restricted synthetic peptide P18I10 (RGPGRAFVTI, corresponding to residues 318–327 of the gp160 envelope protein of HIV-1 [IIIB]) [31, 32] or a control peptide MCMVpp89 [33] from murine cytomegalovirus (YPHFMPTNL, H-2L^d-restricted) dissolved in serum-free complete DMEM. Cells were washed three times, and 2×10^4 B4.2.3 T-hybridoma cells (H-2D^d-restricted, p18I10-specific) [9] in 200 μl of 10% FCS complete DMEM were added to each well and the plates were incubated at 37 °C overnight. IL-2 in the culture medium was measured by commercially available ELISA assay (InterTest Mouse IL-2 ELISA Kit, Genzyme). Briefly, culture supernatants were added to wells precoated with polyclonal anti-mouse IL-2. After 1 h at 37 °C, bound IL-2 was detected with biotinylated anti-IL-2, followed by streptavidin-peroxidase. Absorbance of oxidized tetramethylbenzidine was measured at 450 nm.

2.6 Radiolabeling and immunoprecipitation of membrane-bound proteins

After harvesting by trypsinization, 2×10^7 cultured cells were washed three times with PBS by centrifuging 5 min at 1500 rpm and room temperature. Cells (>95% viable by trypan blue exclusion) were brought to 1×10^8 cells/ml in supplemented PBS. Cell surface labeling with carrier-free ^{125}I (Amersham) was done at 0 °C with lactoperoxidase [34]. The labeled cells were lysed with 0.5% NP40, 10 mM Tris (pH 7.6), 1.5 mM $MgCl_2$, containing inhibitors 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.02 mM phenylmethylsulfonyl fluoride. Immunoprecipitations on equal aliquots of lysate were performed as described [19], using normal rabbit serum to preclear prior to adding specific antibody. Immunoprecipitates were analyzed by PAGE and fluorography [35].

3 Results

3.1 FACS and immunoprecipitation analysis of surface expression of SCβD^{d}m in transfected DAP3 cells

In initial experiments, we assessed expression of SCβD^{d}m by transfected DAP3 cells. Fig. 2A shows the results of an indirect immunofluorescence assay testing for surface expression of the transfected H-2D^d and of endogenous H-2K^k. Surface expression of correctly folded H-2D^d α1α2 domains was detected with mAb 34-5-8, a mAb that is specific for a conformational epitope of H-2D^d [16] (Fig. 2A). Anti H-2D^d α3 domain (34-2-12) gave similar staining (data not shown). Only background staining was seen with negative control antibody 28-14-8 (anti-L^d) or no first antibody. The positive control was mAb 11-4-1 (anti-endogenous H-2K^k). Untransfected control DAP3 cells were only positive when stained with mAb 11-4-1 (Fig. 2B).

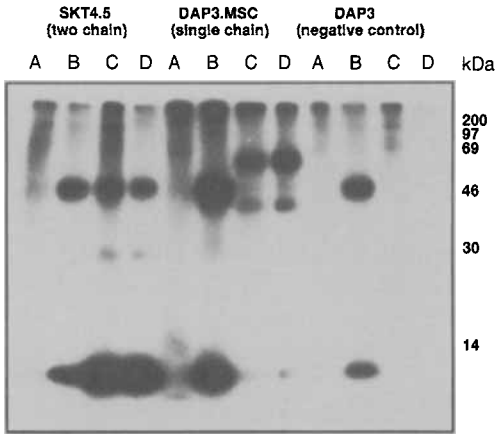

Figure 3. SCβD^{d}m is an approximately 60-kDa cell surface protein without separable β_2m. Transfected DAP3 cells and control cells were surface-labeled with ^{125}I by lactoperoxidase reaction. Equal aliquots of labeled lysates were immunoprecipitated. (A) No first mAb (negative control). (B) 11-4-1 (anti-H-2K^k, endogenous), (C) 34-2-12 (anti-H-2D^d α3), and (D) 34-5-8 (anti-H-2D^d α1α2).

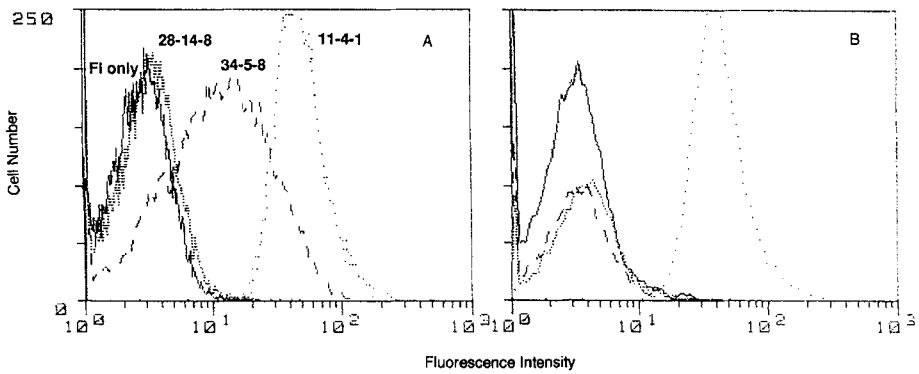

Figure 2. Expression of membrane-bound single-chain class I MHC molecules by SCβD^{d}m-transfected DAP3 cells. Cells were stained with either mAb 34-5-8 (anti-H-2D^d, α1α2), 11-4-1 (anti-H-2K^k endogenous, positive control), or 28-14-8 (anti-H-2L^d, negative control), followed by FITC-conjugated goat F(ab')$_2$ anti-mouse IgG. (A) DAP3.MSC, (B) Untransfected DAP3 cells.

To compare the biochemical structure of the wild-type two-chain and chimeric single-chain proteins expressed by transfected DAP3 cells, the cells were surface-labeled with ^{125}I by the lactoperoxidase method (Fig. 3). Immunoprecipitation of lysates with the mAb 34-5-8 (anti-correctly folded α1α2) and 34-2-12 (anti-α3) gave a main band at the expected size of approximately 60 kDa (DAP3.MSC, lanes C and D). The additional broad band at about 40 kDa in these lanes may represent partially degraded SCβD^{d}m, or be due to trypsinization prior to labeling. A trace of a 12-kDa band that might include liberated β_2m (DAP3.MSC, lanes C, D) was also seen. Densitometry (data not shown) found it to be less than one percent of the amount of β_2m complexed to a similar amount of H-2D^d heavy chain in the two-chain H-2D^d, lanes C and D. The control cell line SKT4.5 (expressing wild-type two-chain H-2D^d on DAP3 surface) showed the expected free H-2D^d heavy chain of about 45 kDa. The mAb 11-4-1 precipitated endogenous H-2K^k heavy chain from each cell line. (The greater intensity of the H-2K^k band relative to the β_2m band may be because there are three more tyrosine residues in H-2K^k than in H-2D^d [36].) Thus, cells transfected with the SCβD^{d}m gene express cell surface molecules that have the serological phenotype of H-2D^d, and the molecular phenotype of the expected single-chain molecule.

3.2 Antigen presentation by SCβD^{d}m-transfected DAP3 cells

To test the functional ability of SCβD^{d}m in antigen presentation, transfected and untransfected control cells were assayed for their ability to present a synthetic peptide to a peptide-specific H-2D^d-restricted T cell hybridoma. Cells were pulsed with various amounts of the H-2D^d-binding ten-residue peptide p18I10 from HIV gp160 [31, 32], or with no peptide. The H-2D^d-restricted, p18I10-specific T cell hybridoma B4.2.3 cells were then added, incubated overnight at 37 °C, and culture supernatants tested for the presence of IL-2. As shown in Fig. 4, DAP3.MSC cells bearing SCβD^{d}m molecules effectively presented the peptide antigen to the T hybridoma, with a half-maximal stimulation at a peptide concentration of about 2×10^{-10} M, comparable to cells bearing the wild-type two-chain H-2D^d. Neither untransfected cells (Fig. 4), nor cells pulsed with no peptide ("0" on the X axis), nor cells pulsed with a control H-2L^d-restricted peptide MCMVpp89 (data not shown) had an effect. This result suggests that not only is the SCβD^{d}m molecule synthesized, correctly folded and expressed, but that it is also capable of binding a peptide antigen and of specifically interacting with the appropriate T cells.

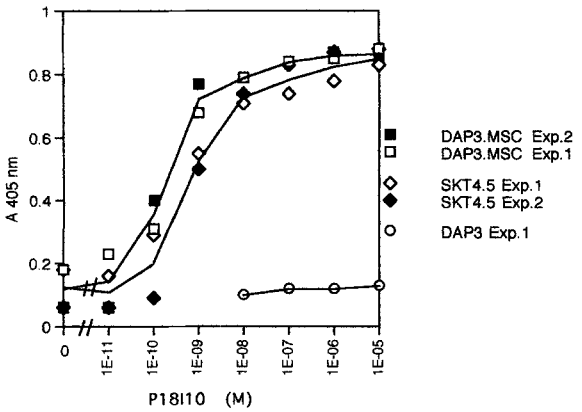

Figure 4. Antigen presentation by DAP3 cells transfected with SCβD^{d}m, as measured by stimulation of IL-2 production. DAP3.MSC, SKT4.5 cells (expressing two-chain D^d), and untransfected DAP3 cells were incubated with peptide p18I10, or with no peptide at 37 °C for 3 h. After addition of T hybridoma B4.2.3 cells and overnight incubation, 100 µl of culture supernatant from each well was assayed for IL-2 by a capture ELISA (see Sect. 2.5). Two experiments are shown. Values plotted are means of duplicates from the individual experiments, and the dose response lines drawn through the average of the two experiments. Under the conditions of the assay, 1 ng/ml of mouse IL-2 gave an absorbance of about 0.5.

3.3 Effect of peptide and low temperature on SCβD^{d}m expression and antigen presentation in cells deficient for peptide transport

Although SCβD^{d}m is expressed and functional on DAP3 cells, we would like to test the immune response to SCβD^{d}m molecules homogenously loaded with single antigenic peptides. Uniform loading of exogenous peptide may be achieved using the "empty" class I molecules of cells that are deficient in peptide transport into the endoplasmic reticulum [37, 38]. Therefore, we transfected the SCβD^{d}m construct into EE2H3 cells, in which it had been previously found that expression of a transfected H-2D^d gene was markedly enhanced by the addition of an appropriate antigenic peptide, or by culturing cells at low temperature [16]. EE2H3 cells transfected with SCβD^{d}m were incubated at 27 °C or 37 °C overnight, with or without p18I10 peptide. In the absence of added peptide, there was almost no SCβD^{d}m found on the cell surface at physiological temperature. This could have been due to the peptide transport deficit of the EE2H3 cells, or to lack of expression of the transfected construct. However, the SCβD^{d}m construct was indeed adequately expressed because greatly enhanced surface expression was seen when cells were exposed to both peptide and low temperature (Fig. 5).

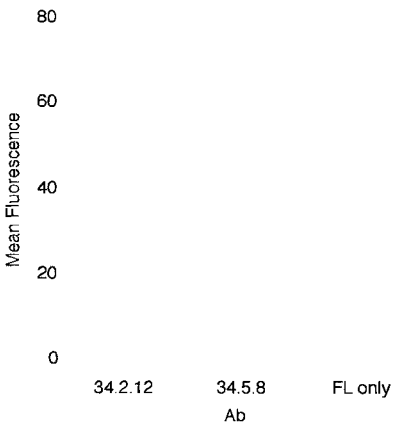

Figure 5. Stabilization of SCβD^{d}m surface expression on EE2H3 cells by peptide p18I10 and low temperature. EE2H3.MSC cells were exposed to medium alone (complete DMEM 10% FCS) or medium containing 10^{-4} M peptide p18I10 for 12 h at 37 °C or 27 °C, then analyzed for the 34-5-8 and 34-2-12 epitopes by indirect immunofluorescence.

Enhanced surface expression of SCβD^{d}m on addition of peptide was seen even at 37 °C. At 27 °C without peptide, there was more expression of the α3 epitope detected by mAb 34-2-12, than there was of the α1α2 epitope detected by mAb 34-5-8. These results suggest, as has been found for two-chain class I molecules [16, 39], that "empty" SCβD^{d}m molecules (or SCβD^{d}m molecules bound to low-affinity peptides) reach the surface of transfected peptide transport-defective cells, where the peptide binding site can be stabilized by added peptide.

To determine whether "empty" SCβD^{d}m on the surface of EE2H3 cells could present exogenously added peptide p18I10 to the B4.2.3 T cell hybridoma, a T cell stimulation followed by IL-2 ELISA assay was performed (Fig. 6).

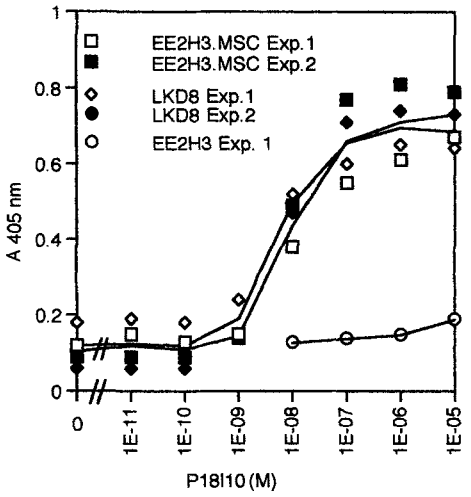

Figure 6. Antigen presentation by EE2H3 cells transfected with SCβD^{d}m, as measured by IL-2 ELISA. EE2H3.MSC cells, LKD8 cells (EE2H3 expressing two-chain D^d), and untransfected EE2H3 cells were assayed as described in the legend to Fig. 4.

After exposing SCβD^{d}m-transfected EE2H3 cells to peptide p18I10 at 37 °C, they were able to stimulate IL-2 secretion by the B4.2.3 T hybridoma cells (Fig. 6), with half maximal stimulation at a peptide concentration of 9×10^{-9} M, comparable to LKD8 cells expressing wild-type two-chain H-2D^d [16]. These data suggest that the "empty" single chain class I molecules are able to bind and present exogenous peptides to T hybridoma cells.

3.4 Transfected SCβD^{d}m is expressed in the absence of endogenous β_2m, but does not rescue expression of endogenous class I molecules

In order to determine if the single-chain class I MHC molecules were using endogenous β_2m, rather than their own covalently linked β_2m domain for assembly, we transfected SCβD^{d}m DNA into the β_2m-negative human cell line KJ29 [30]. Stable transformed cells were cloned. Flow cytometry analysis (Fig. 7A) showed surface expression of correctly folded SCβD^{d}m molecules at 37 °C. The ^{125}I-labeling and immunoprecipitation indicated that an approximately 60-kDa SCβD^{d}m protein with mAb 34-5-8 reactivity was present on the surface of the transfected

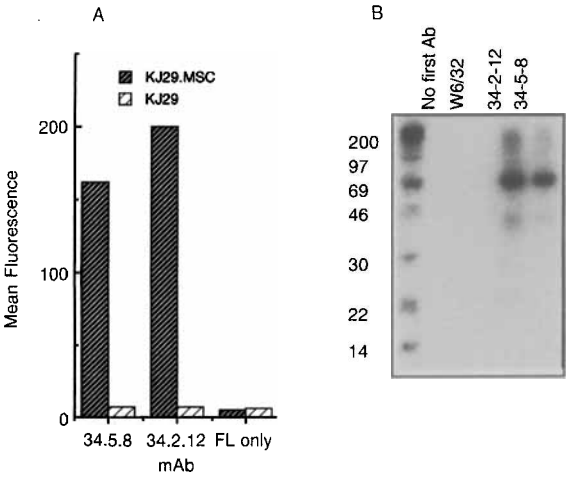

Figure 7. Surface expression of transfected SCβD^{d}m in the β_2m-negative human cell line KJ29. (A) The level of SCβD^{d}m on transfected KJ29 cloned cell line KJ29.MSC and control KJ29 was assayed by indirect immunofluorescence with mAb 34-5-8 and 34-2-12. (B) ^{125}I-surface labeling. The transfected KJ29 cloned cell line KJ29.MSC was surface-labeled with ^{125}I by lactoperoxidase. Labeled lysates were immunoprecipitated with mAb W6/32 (anti-HLA-A, B, C), 34-2-12 (anti-H-2D^d α3), 34-5-8 (anti-H-2D^d α1α2) or no first mAb (negative control).

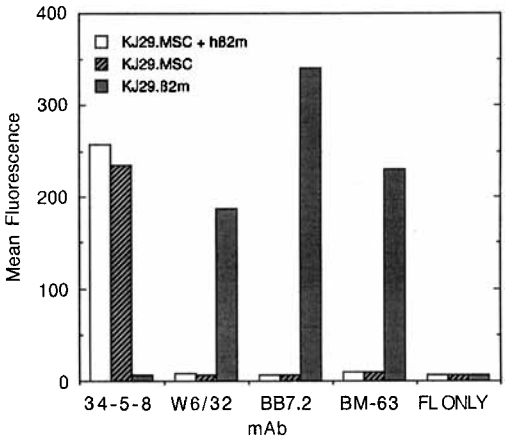

Figure 8. Class I HLA expression is not rescued by the β_2m domain of SCβD^{d}m or exogenous human β_2m, but is rescued by transfected human β_2m cDNA. KJ29 cells transfected with SCβD^{d}m (KJ29.MSC) were incubated in serum-free DMEM at 37 °C overnight in the presence or absence of extracellular human β_2m, 35 μg/ml (3×10^{-6} M), followed by reaction with anti-class I HLA W6/32 and BB7.2, anti-human β_2m BM-63, or anti-H-2D^d 34-5-8, by indirect immunofluorescence. KJ29 cells transfected with human β_2m (KJ29.β_2m) were reacted with same mAb.

KJ29 cells, while assembled HLA class I molecules were not found with mAb W6/32 (anti-HLA-A, B, C) (Fig. 7B). HLA class I MHC molecules were also undetectable with mAb BB7.2 (anti-HLA-A2.1 α1) and BM-63 (anti-human β_2m) by flow cytometry (data not shown). This result indicates that SCβD^{d}m does not need free endogenous β_2m for expression. It also indicates that the β_2m domain of SCβD^{d}m did not rescue expression of the endogenous HLA class I molecules. Similarly, endogenous HLA expression was not rescued when the SCβD^{d}m-transfected β_2m-

negative KJ29 cells (KJ29.MSC) were exposed to exogenous human β_2m (Fig. 8). On the other hand, the positive control KJ29.β_2m (transfected with human β_2m) did have HLA class I surface expression (Fig. 8).

4 Discussion

As a preliminary step towards using full-length cell surface single-chain class I MHC molecules as T cell vaccine components, we have constructed a recombinant gene encoding such a molecule, SCβD^{d}m, which on expression is correctly folded and functional in antigen presentation to the appropriate T hybridoma. Because our previously studied soluble single-chain class I MHC molecule (SCβD^{d}s) was actively secreted by transfected DAP3 cells [19], we first made use of DAP3 cells to investigate the full-length membrane-bound form of the single-chain class I H-2D^d molecule, SCβD^{d}m. We show correct folding and surface expression of SCβD^{d}m on the DAP3 cells by indirect immunofluorescence (Fig. 2) and immunoprecipitation (Fig. 3).

Effective antigen-presentation by SCβD^{d}m molecules, comparable to that of wild-type two-chain molecules, was shown by the analysis of the H-2D^d-restricted p18I10 peptide-specific T hybridoma response (Figs. 4 and 6). This provides further evidence that SCβD^{d}m is correctly folded into the same conformation as the wild-type two-chain H-2D^d. This is a desirable property if single-chain class I molecules are to be used to generate CTL responses that can react with wild-type two-chain class I molecules on the surface of infected cells.

Our experiments also showed that both low temperature and extracellular peptide p18I10 can stabilize SCβD^{d}m molecules that reach the surface of peptide transport-deficient EE2H3 cells at 27 °C and 37 °C (Fig. 5), in a form recognizable by the appropriate T hybridoma cells (Fig. 6). These stabilization and antigen presentation data indicate that the peptide binding site of the "empty" SCβD^{d}m molecules is not "locked up" by the non-dissociable β_2m domain, and is capable of binding added peptide, as has been found for the soluble single-chain molecules [19–22]. Extracellular peptide pulsing can stabilize some surface expression even at 37 °C. This suggests (as is the case for the wild-type two-chain class I molecules) that peptide transport-deficient cells may be used to achieve high efficiency peptide loading of full-length single-chain molecules, a necessary step prior to their use as potential T cell immunogens.

The formal possibility remained that the SCβD^{d}m molecule might rely on endogenous β_2m instead of its own covalently linked β_2m domain for folding into the native conformation. For this reason, we transfected the SCβD^{d}m plasmid into the β_2m-negative human cell line KJ29 [30]. If single-chain molecules had to use endogenous β_2m to achieve a stable conformation, they would not have been expressed by the β_2m-negative KJ29 cells. However, immunofluorescence (Fig. 7A) and immunoprecipitation (Fig. 7B) indicated that the transfected β_2m-negative KJ29 cells cultured at 37 °C did express correctly folded SCβD^{d}m. This shows that the single-chain class I molecules require no other source of β_2m to achieve correct folding.

We took advantage of the β_2m-negative KJ29 cells to investigate whether the β_2m domain of SCβD^{d}m could act (catalytically or stoichiometrically) to help new class I heavy chains to fold and form heavy chain-peptide complexes. Such complexes, if they reached the cell surface, might be further stabilized by extracellular β_2m. However, the SCβD^{d}m-transfected KJ29 cells remain class I HLA-negative when incubated at 37 °C in the presence of added extracellular human β_2m (Fig. 8), as well as at 27 °C (data not shown), indicating that the covalently linked β_2m domain of SCβD^{d}m did not play such a role. That this failure is not due to lack of HLA class I gene expression is shown by the rescue of such expression at 37 °C by transfected β_2m (Fig. 8). When heavy chain and β_2m are synthesized in peptide transport-deficient cells, "empty" molecules reach the surface that can be stabilized by the addition of extracellular peptide [14, 16, 37–40]. However, when heavy chain was synthesized in the absence of β_2m, even in a cell such as KJ29, which is competent for peptide transport into the ER (as evidenced by the stable expression of transfected SCβD^{d}m), surface expression of a heavy chain-peptide complex could not be rescued by extracellular β_2m (Fig. 8). This suggests that in the absence of endogenous β_2m, an HLA heavy chain-peptide complex either did not form and reach the surface (although it can be demonstrated [41] for H-2D^b), or that only traces of heavy chain reach the surface in a rescueable form that can be detected by CTL [42]. Traces of heavy chain on the surface of β_2m "knockout" mice have also been detected by CTL that do not cross-react with β_2m-positive class I molecules [42].

In conclusion, the present studies indicate that engineered full-length single-chain MHC molecules are not only effectively expressed in a functionally competent conformation at the cell surface, but that the control of their expression is *cis* dominant. Thus, the possibility exists that not only cells (as in this study), but also transgenic mice expressing only a single type of cell surface MHC molecule can be engineered. Such cells and animals may prove useful for examining the role of individual MHC molecules in immune responses.

We express our appreciation to Drs. Patricio Giacomini and Michele Fiscella for generously making the KJ29.β_2m cell line available to us prior to publication.

Received April 20, 1994; in revised form July 25, 1994; accepted July 26, 1994.

5 References

1 Braciale, T. J. and Braciale, V. L., *Semin. Immunol.* 1992. *4:* 81.
2 Townsend, A. R. M., Bastin, J., Gould, K. and Brownlee, G., *Nature* 1986. *324:* 575.
3 Falk, K., Rotzschke, O., Deres, K., Metzger, J., Jung, G. and Rammensee, H. G., *J. Exp. Med.* 1991. *174:* 425.
4 Van Bleek, G. M. and Nathenson, S. G., *Nature* 1990. *348:* 213.
5 Williams, D. B., Barber, B. H., Flavell, R. A. and Allen, H., *J. Immunol.* 1989. *142:* 2796.
6 Krangel, M. S., Orr, H. T. and Strominger, J. L., *Cell* 1979. *18:* 979.
7 Ribaudo, R. K. and Margulies, D. H., *J. Immunol.* 1992. *149:* 2935.

8 Townsend, A., Elliot, T., Cerundolo, V., Foster, L., Barber, B. and Tse, A., *Cell* 1990. *62:* 285.
9 Kozlowski, S., Takeshita, T., Boehncke, W.-H., Takahashi, H., Boyd, L. F., Germain, R. N., Berzofsky, J. A. and Margulies, D. H., *Nature* 1991. *349:* 74.
10 Rock, K. L., Rothstein, L. E., Gamble, S. R. and Benacerraf, B., *Proc. Natl. Acad. Sci. USA* 1990. *87:* 7517.
11 Rock, K. L., Gamble, S., Rothstein, L. and Benacerraf, B., *Proc. Natl. Acad. Sci. USA* 1991. *88:* 301.
12 Vitiello, A., Potter, T. A. and Sherman, L. A., *Science* 1990. *250:* 1423.
13 Boyd, L. F., Kozlowski, S. and Margulies, D. H., *Proc. Natl. Acad. Sci. USA* 1992. *9:* 2242.
14 Rock, K. L., Gramm, C. and Benacerraf, B., *Proc. Natl. Acad. Sci. USA* 1991. *88:* 4200.
15 Hansen, T. H., Myers, N. B. and Lee, D. R., *J. Immunol.* 1988. *140:* 3522.
16 Otten, G. R., Bikoff, E., Ribauo, R. K., Kozlowski, S., Margulies, D. H. and Germain, R. N., *J. Immunol.* 1992. *148:* 3723.
17 Godeau, F., Casanova, J. L., Luescher, I. F., Fairchild, K. D., Delarbre, C., Saucier, C., Gachelin, G. and Kourilsky, P., *Int. Immunol.* 1992. *4:* 265.
18 Koller, B. H. and Smithies, O., *Proc. Natl. Acad. Sci. USA* 1989. *86:* 8932.
19 Mage, M. G., Lee, L., Ribaudo, R. K., Corr, M., Kozlowski, S., McHugh, L. and Margulies, D. H., *Proc. Natl. Acad. Sci. USA* 1992. *89:* 10658.
20 Mottez, E., Jaulin, C., Godeau, F., Choppin, J., Levy, J. P. and Kourilsky, P., *Eur. J. Immunol.* 1991. *27:* 467.
21 Abastado, J.-P., Ojcius, D. M., Casrouge, A., Yeh, P., Schumacher, T. N. M., Ploegh, H. and Kourilsky, P., *Eur. J. Immunol.* 1993. *23:* 1776.
22 Ojcius, D. M., Abastado, J. P., Casrouge, A., Mottez, E., Cabanie, L. and Kourilsky, P., *J. Immunol.* 1993. *151:* 6020.
23 Ozato, K., Mayer, N. M. and Sachs, D. H., *Transplantation* 1982. *34:* 113.
24 Evans, G. A., Margulies, D. H., Shykind, B., Seidman, J. G. and Ozato, K., *Nature* 1982. *300:* 755.
25 Ozato, K., Hansen, T. H. and Sachs, D. H., *J. Immunol.* 1980. *125:* 2473.
26 Oi, V. T., Jones, P. P., Goding, J. W. and Herzenberg, L. A., *Curr. Top. Microbiol. Immunol.* 1978. *81:* 115.
27 Brodsky, F. M., Parham, P., Barnstable, C. J., Crumpton, M. J. and Bodmer, W. F., *Immunol. Rev.* 1979. *47:* 3.
28 Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. and Pease, L. R., *Gene* 1989. *77:* 61.
29 Gunning, P., Leavitt, J., Muscat, G., Ng, S.-Y. and Kedes, L., *Proc. Natl. Acad. Sci. USA* 1987. *84:* 831.
30 del Senno, L., Anzanel, D., Barbieri, R., Buzzoni, D., Rossi, P., Piva, R. and Gambari, R., *Cell Biol. Int. Rep.* 1986. *10:* 195.
31 Takahashi, H., Cone, J., Cease, A. K., Houghton, R., Cornet, J. L., deLisi, C., Moss, B., Germain, R. N. and Berzofsky, J. A., *Proc. Natl. Acad. Sci. USA* 1988. *85:* 3105.
32 Shirai, M., Pendleton, C. D. and Berzofsky, J. A., *J. Immunol.* 1992. *148:* 1657.
33 Reddehase, M. J., Rothbard, J. B. and Koszinowski, U. H., *Nature* 1989. *337:* 651.
34 Samelson, L. E., in *Current Protocols in Immunology,* J. Wiley & Sons, New York 1991, p. 8.11.1.
35 Margulies, D. H., Ramsey, A. L., Boyd, L. F. and McClusky, J., *Proc. Natl. Acad. Sci. USA* 1986. *83:* 5252.
36 Kabat, E. A. et al., *Sequences of proteins of immunological interest* (NIH Publication No. 91-3242), Vol. I, 5th Edn., NIH, Bethesda 1991.
37 Jackson, M. R., Song, E. S., Yang, Y. and Peterson, P. A., *Proc. Natl. Acad. Sci. USA* 1992. *89:* 12117.
38 Kozlowski, S., Corr, M., Takeshita, T., Boyd, L. F., Pendleton, C. D., Germain, R. N., Berzofsky, J. A. and Margulies, D. H., *J. Exp. Med.* 1992. *175:* 1417.
39 Ljunggren, H. G., Stam, N. J., Ohlen, C., Neefjies, J. J., Hoglund, P. L., Heemels, M. T., Bastin, J., Schumacher, T. N. M., Townsend, A., Karre, K. and Ploegh, H., *Nature* 1990. *346:* 476.
40 Catipovic, B., Dal Porto, J., Mage, M., Johansen, T. E. and Schneck, J. P., *J. Exp. Med.* 1992. *176:* 1611.
41 Bix, M. and Raulet, D., *J. Exp. Med.* 1992. *176:* 829.
42 Glas, R., Franksson, L., Ohlen, C., Hoglund, P., Koller, B., Ljunggren, H. and Karre, K., *Proc. Natl. Acad. Sci. USA* 1992. *89:* 11381.
43 Margulies, D. H., Evans, G. A., Ozato, K., Camerini-Otero, R. D., Tanaka, K., Appella, E. and Seidman, J. G., *J. Immunol.* 1983. *130:* 463.
44 Silverman, T., Rein, A., Orrison, B., Langloss, J., Bratthauer, G., Miyazaki, J. and Ozato, K., *J. Immunol.* 1988. *140:* 4378.