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Immunology

A recombinant, soluble, single-chain class I major histocompatibility complex molecule with biological activity

(H-2D^d/β_2-microglobulin/antigen presentation)

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ABSTRACT **Heterodimeric class I major histocompatibility complex molecules, which consist of a 45-kDa heavy-chain and a 12-kDa β_2-microglobulin (β_2m) light chain, bind endogenously synthesized peptides for presentation to antigen-specific T cells. We have synthesized a gene encoding a single-chain, soluble class I molecule derived from mouse H-2D^d, in which the carboxyl terminus of β_2m is linked via a peptide spacer to the amino terminus of the heavy chain. The chimeric protein is secreted efficiently from transfected L cells, is thermostable, and when loaded with an appropriate antigenic peptide, stimulates an H-2D^d-restricted antigen-specific T-cell hybridoma. Thus, functional binding of peptide does not require the complete dissociation of β_2m, implying that a heavy chain/peptide complex is not an obligate intermediate in the assembly of the heavy-chain/β_2m/peptide heterotrimer. Single-chain major histocompatibility complex molecules uniformly loaded with peptide have potential uses for structural studies, toxin or fluor conjugates, and vaccines.**

Balanced production of naturally multimeric proteins (such as antibodies) can be facilitated by engineering them as single-chain molecules (1–3). Such a strategy links the structural domains so they cannot dissociate and may be an initial step to the production of toxin conjugates and large-scale synthesis in bacteria. Analysis of the kinetics and thermodynamics of folding, domain interaction, and ligand binding by such tethered molecules may also provide insight into the conformational changes that accompany these events. Because the heterodimeric major histocompatibility complex (MHC) class I molecules play an important role in immune responses, we have engineered a single-chain MHC class I molecule, in which the immunoglobulin domain-like subunit β_2-microglobulin (β_2m) is linked via a peptide spacer to the amino terminus of the peptide-binding H-2D^d heavy chain (SCβD^d_s). A similar approach, in which the carboxyl terminus of the extracellular portion of a truncated H-2K^d heavy chain was linked to the amino terminus of β_2m through a spacer, has been reported (4), but those molecules were neither expressed efficiently nor secreted and were not shown to function in the activation of T cells.

The class I MHC molecules, synthesized by and expressed on the surfaces of most vertebrate cells, consist of a 45-kDa heavy chain, noncovalently associated with a 12-kDa light chain, β_2m. These MHC class I molecules bind peptides derived from endogenously synthesized self- (5) or pathogen-encoded proteins (6–9) and display them for recognition by T lymphocytes through their $\alpha\beta$ antigen-specific T-cell receptors. The β_2m chain plays an important role in MHC class I intracellular transport (10–12), peptide binding (13–18), and conformational stability (12, 19–22). 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 biosynthetic maturation and cell-surface expression (16, 17, 23).

The order of assembly of the heterotrimer is not known. There are data consistent with (*i*) heavy chain first binding to β_2m and the complex then binding peptide or (*ii*) heavy chain first binding peptide and the complex then being stabilized by β_2m (24, 25). We hypothesized that studies of single-chain molecules in which β_2m was covalently tethered to the class I heavy chain might help determine whether normal formation of the ternary complex requires a heavy chain/peptide intermediate, or whether a heavy chain/β_2m complex can form and then bind peptides.

MATERIALS AND METHODS

Antibodies. Hybridomas producing monoclonal antibody (mAb) 34-5-8, which binds to a conformationally labile and peptide-dependent epitope of the $\alpha_1\alpha_2$ domain of H-2D^d, and mAb 34-2-12, which binds to a more stable and peptide-independent epitope of the α_3 domain (22, 26, 27), were from American Type Culture Collection. Polyclonal antibodies to H-2D^d heavy chain (322-1) and to murine β_2m (322-3) were produced by immunizing rabbits with denatured heavy chain and β_2m, respectively. The anti-heavy-chain antibody has a preference for free heavy chains and precipitates little or no heterodimeric heavy chain/β_2m complex.

Construction of the Single-Chain β_2m/H-2D^d Molecule. Genes for β_2m and class I MHC heavy chain were linked so as to connect the carboxyl terminus of β_2m to the amino terminus of the heavy chain via a peptide spacer. The length of the spacer needed was measured on a three-dimensional computer graphic model of the H-2D^d murine β_2m heterodimer, based on the high-resolution crystallographic coordinates (Brookhaven Protein Data Bank no. 3hla) of the human class I molecule HLA-A2 (28), by amino acid substitution and multiple rounds of energy minimization using QUANTA version 3.0 (Polygen, Waltham, MA). These measurements indicated that a 15-amino acid peptide spacer consisting of the sequence (GGGGS)$_3$ (29) would allow β_2m to find its normal position relative to the heavy chain. The β_2m-encoding gene was amplified by PCR from a cDNA clone (pβ2m.1), which was constructed from a cDNA library derived from C57BL6 mouse liver. Its structure was confirmed by DNA sequencing (R.K.R. and D.H.M., unpublished work). We used a 5′-sense primer 3504 (TGG GTC GAC ATG GCT CGC TCG GTG ACC CTG) containing the sequence for a *Sal* I site immediately 5′ to the signal peptide

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Abbreviations: MHC, major histocompatibility complex; β_2m, β_2-microglobulin; H-2D^d_s, soluble analogue of H-2D^d; SCβD^d_s, soluble single-chain class I MHC molecule that contains β_2m/spacer/H-2D^d_s.

sequence and a 3′ antisense primer 3505 (GCC GCC ACC CGA GCC GCC TCC GCC CGA ACC GCC ACC TCC CAT GTC TCG ATC CCA GTA GAC) containing the sequence for the 3′ end of β_2m and for a portion of the peptide spacer. The heavy-chain gene was PCR-amplified from a plasmid containing cDNA encoding a soluble form of H-2D^d (H-2D^d_s) consisting of the α_1, α_2, and α_3 H-2D^d domains and the 27 carboxyl-terminal amino acid residues of Q10^b (14), using a 5′ sense primer 3506 (GGC GGC TCG GGT GGC GGC GGC TCT GGC GGA GGT GGA TCC GGC TCA CAC TCG CTG AGG) encoding a portion of the peptide spacer, an overlap to the 3′ primer of β_2m, sequence for the 5′ end of the H-2D^d gene, and a 3′ antisense primer 3507 (ACT AAG CTT CAC TGG AGC GGG AGC ATA GTC) containing H-2D^d 3′-untranslated region sequence and a *Hin*dIII site. The two PCR products were mixed and spliced by overlap extension (30), using the 5′ primer for β_2m and the 3′ primer for H-2D^d. The PCR product was purified, cut with *Sal* I and *Hin*dIII, repurified, and ligated into a eukaryotic expression vector pHβAPr-1 neo (31) by standard methods. DNA sequencing revealed three point mutations, probably PCR-induced, which were corrected by site-directed mutagenesis using the Altered Sites kit from Stratagene. The corrected SCβD^d_s clone (for a soluble single-chain class I MHC molecule that contains β_2m/spacer/H-2D^d_s) was sequenced in its entirety with Sequenase 2 (United States Biochemical).

Transfection. DAP-3 cells were exposed to DNA precipitated with calcium phosphate (32). Transformants were selected with the antibiotic G418 and cloned by limiting dilution.

ELISA of Secreted Class I Proteins. Immulon 4 (Dynatech) 96-well flat-bottom plates (precoated with an affinity-purified hamster anti-mouse H-2 antibody GBH6) were incubated overnight at 4°C with culture supernatants, washed, and treated sequentially with either mAb 34-2-12 or mAb 34-5-8, followed by alkaline phosphatase-conjugated rat anti-mouse immunoglobulin (14). Molar concentrations were calculated relative to a reference preparation of affinity-purified two-chain H-2D^d. All values are mean ± SEM of three determinations.

Radiolabeling and Immunoprecipitation of Cells. Transfected DAP-3 cells in 24-well plates were pulsed for 16 hr with 900 μCi of [^{35}S]methionine per ml (1 Ci = 37 GBq). Culture supernatants were precleared with normal rabbit serum, and aliquots were then incubated with antibody, followed by protein A-Sepharose beads. Washed beads were boiled with 2% SDS/0.35 M 2-mercaptoethanol, and supernatants were analyzed by PAGE and fluorography (33).

Immunoblotting. Soluble single-chain class I MHC molecules (from SCβD^d_s clone) were affinity-purified at 4°C by passing culture supernatants through a column of mAb 34-2-12 coupled to Sepharose 4B and eluting with carbonate buffer (0.15 M NaCl/0.15 M Na_2CO_3) at pH 11.5. Fractions (1.0 ml) were collected in tubes containing 0.25 ml of 2 M Tris·HCl, pH 7.6, to get prompt neutralization. The purified SCβD^d_s and soluble two-chain H-2D^d_s were electrophoresed after denaturation and reduction on a 20% Phast Gel (Pharmacia) under denaturing conditions and blotted onto nitrocellulose. The blot was blocked with 1% dry milk, incubated with rabbit anti-β_2m (322-3), and developed with ^{125}I-labeled protein A (Amersham).

T-Cell Stimulation Assays. Affinity-purified single-chain SCβD^d_s or two-chain H-2D^d_s molecules were coated onto a Dynatech Immulon 4 plate at 5 μg/ml for 2–3 hr at 27°C. Both the single-chain and two-chain molecules were purified from cell culture supernatants containing fetal calf serum. Two-chain H-2D^d_s, grown under these conditions, has a limited dependence on exogenous β_2m (S.K. and D.H.M., unpublished work). After washing with phosphate-buffered saline, the plates were blocked for 2 hr at 37°C with 1% fetal calf serum/Dulbecco's modified Eagle's medium, washed again, and incubated overnight at 37°C with peptide p18I10 (Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile) from human immunodeficiency virus III$_B$ glycoprotein 120 envelope (ref. 34; T. Takeshita, S.K., R. D. England, R. Brower, H. Takahashi, C. DeLisi, C. D. Pendleton, D.H.M., and J. A. Berzofsky, personal communication) dissolved in phosphate-buffered saline. After three more washes, 2 × 10^4 B4.2.3 T-hybridoma cells (14) in 200 μl of Dulbecco's modified Eagle's medium/10% fetal calf serum/2 mM glutamine/nonessential amino acids/gentamycin at 50 μg/ml/50 μM 2-mercaptoethanol were added to each well. Plates were again incubated overnight at 37°C. The next day the cells were pulsed with 1 μCi of [^{3}H]thymidine and harvested; incorporated label was counted after a further 6 hr at 37°C. Percent inhibition of growth was calculated as [(cpm of T-hybridoma cells in the presence of peptide and purified H-2D^d_s) ÷ (cpm of T-hybridoma cells in the absence of peptide with purified H-2D^d_s)] × 100.

RESULTS

Construction and Expression of a Gene Encoding a Single-Chain Class I Molecule. We designed our construct to encode β_2m amino terminal to H-2D^d_s, linked via an amino acid spacer, so that the synthesis and folding of the compact, immunoglobulin-like domain structure of β_2m would precede that of the heavy chain (Fig. 1). We prepared the chimeric gene from a β_2m cDNA clone and H-2D^d_s (14) by using PCR and splicing by overlap extension (30). The *in vitro* recombinant gene fragment SCβD^d_s, encoding the β_2m leader peptide, the β_2m coding block, a 15-amino acid residue spacer, and the H-2D^d_s coding block was cloned in a vector (pHβAPr-1 neo) under control of the human β-actin promoter (31). The resultant recombinant plasmid (pSCβD^{d}15), encoding a single-chain molecule (hereafter called SCβD^d_s) with covalently linked β_2m and H-2D^d_s sequences, was used to stably transfect the murine L cell line DAP-3 (32).

We examined cultures of transfected DAP-3 cells for secretion of SCβD^d_s with a capture ELISA assay and detected material that reacted with mAb 34-5-8, which binds an epitope of the $\alpha_1\alpha_2$ domain unit, as well as with mAb 34-2-12, which sees the α_3 domain (26, 27). Concentrations of up to 1 × 10^{-8} M (0.6 μg/ml) per week accumulated in the supernatant (Fig. 2*A*), and the SCβD^d_s protein was affinity-purified (33). The relative ratio of mAb 34-5-8 to mAb 34-2-12 epitope for SCβD^d_s was higher than that of the heterodimeric H-2D^d_s, indicating that correctly folded molecules were being secreted (Fig. 2*B*) and that the $\alpha_1\alpha_2$ domain of the SCβD^d_s molecules may be more conformationally homogeneous than in two-chain H-2D^d_s molecules. This higher ratio may reflect a greater proportion of correctly folded molecules in the SCβD^d_s preparation. Secreted SCβD^d_s was thermally stable (Fig. 2*C*). This result suggested that it had bound endogenous peptides, inasmuch as "empty" class I MHC molecules are unstable at 37°C (22, 35).

SDS/PAGE Analysis of SCβD^d_s. Having found that correctly folded SCβD^d_s molecules were being secreted, we confirmed that the SCβD^d_s was a single-chain product of the recombinant gene by metabolically labeling SCβD^d_s-secreting cells with [^{35}S]methionine and analyzing culture supernatants by immunoprecipitation and SDS/PAGE. We found a radiolabeled protein with a relative molecular mass under reducing conditions of ≈56 kDa that was immunoprecipitated by a polyclonal rabbit anti-H-2 antiserum 322-1 and by both the anti-α_3 domain 34-2-12 and the anti-$\alpha_1\alpha_2$ domain 34-5-8 mAbs (Fig. 3, single chain, lanes A–C, respectively). Unlike the two-chain H-2D^d_s molecule in which the mAbs coprecipitated the 12-kDa β_2m light chain with the heavy chain (Fig. 3, two chain, lanes B and C), the SCβD^d_s protein showed no copre-

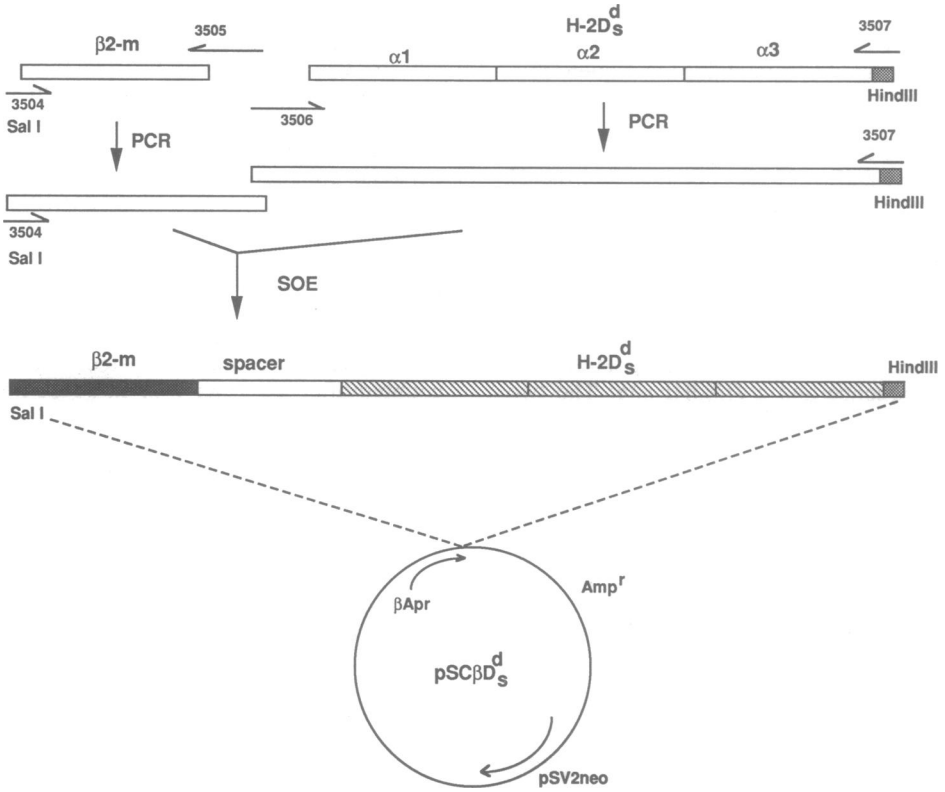

FIG. 1. Map and diagram of the single-chain SCβD^d_s construct. Splicing by overlap extension (30) was used to link the genes so as to connect the carboxyl terminus of β_2m to the amino terminus of the H-2D^d_s heavy chain via a peptide spacer. Also shown are the restriction sites for insertion into the polylinker of pHβAPr-1 neo (βApr) (31).

cipitating β_2m. The difference in size of ≈14 kDa between the SCβD^d_s and the H-2D^d_s heavy chain is consistent with the expected molecular mass of β_2m (12 kDa) plus the 15-amino acid peptide spacer (1.3 kDa). The material that is smaller than 30 kDa (lane B) appears to be an H-2D^d fragment containing the α_3 domain epitope but has not been further characterized. The decreased ability of the anti-α_3 domain mAb to coprecipitate β_2m has been observed consistently (27).

To verify the covalent linkage of β_2m to the heavy-chain, affinity-purified SCβD^d_s was analyzed by SDS/PAGE under reducing conditions (Fig. 4*A*) and by immunoblotting (Fig. 4*B*). SDS/PAGE analysis revealed a major band of ≈56 kDa (Fig. 4*A*, lane 1) and little or no copurifying β_2m light chain. This result contrasts with the H-2D^d_s molecule, which has a heavy chain of 37 kDa associated with the 12-kDa β_2m light chain (Fig. 4*B*, lane 2). Immunoblot analysis of a similar gel using a rabbit anti-β_2m polyclonal antibody (Fig. 4*B*) revealed that the SCβD^d_s 56-kDa band contained β_2m (Fig. 4*B*, lane 1), whereas only the 12-kDa β_2m band of the two-chain molecule reacted with the antibody (lane 2). Thus, the secreted protein product of the SCβD^d_s gene contains β_2m epitopes in a molecule of the anticipated size.

***In Vitro* Peptide Antigen Presentation by SCβD^d_s.** To test for biological activity as an antigen-presenting molecule, affinity-purified SCβD^d_s was pulsed with a synthetic peptide, p18I10, known to be H-2D^d-restricted (34) and tested for the

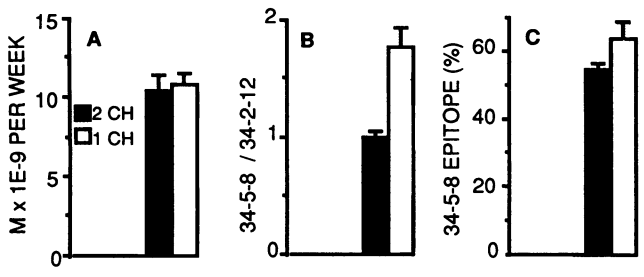

FIG. 2. SCβD^d_s protein secretion, folding, and thermostability. Cell culture supernatants were evaluated for mAb epitopes by ELISA as described. (*A*) Secretion rate. M × 10^{-9} per week. (*B*) Relative ratio of $\alpha_1\alpha_2$ epitope (mAb 34-5-8) to α_3 epitope (mAb 34-2-12) normalized to 1 for H-2D^d_s. (*C*) Percent of mAb 34-5-8 epitope remaining after 45 min at 53°C. □, Single-chain (1 CH) SCβD^d_s; ■, two-chain (2 CH) H-2D^d_s.

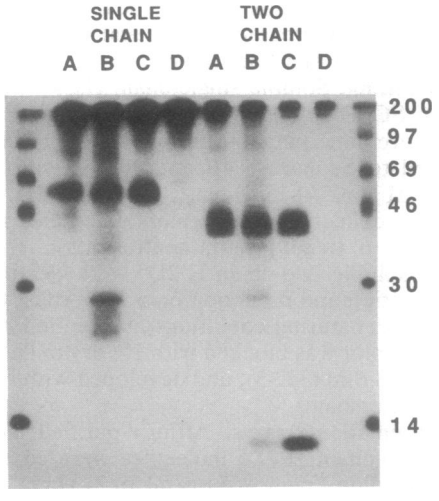

FIG. 3. SCβD^d_s is a secreted protein of 56 kDa without separable β_2m. Radiolabeled culture supernatants were precipitated with rabbit antibody to H-2D^d heavy chain (mAb 322-1) (*A*); mAb 34-2-12 (anti-α_3) (*B*); mAb 34-5-8 (anti-$\alpha_1\alpha_2$) (*C*); or no antibody (*D*). Molecular mass markers are given in kDa. (*Left*) Single-chain SCβD^d_s. (*Right*) Two-chain H-2D^d_s.

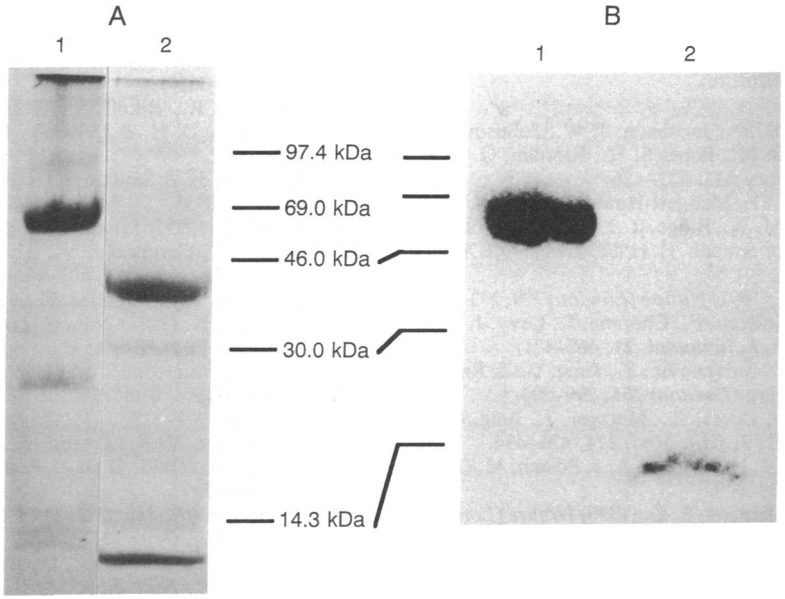

FIG. 4. Affinity-purified SCβD^d_s is not associated with natural β_2m but contains β_2m epitopes. Affinity-purified SCβD^d_s (lanes 1) and affinity-purified soluble two-chain H-2D^d_s (lanes 2) were electrophoresed after denaturation and reduction and analyzed by staining with Coomassie brilliant blue (*A*) or by immunoblotting with rabbit anti-β_2m (322-3) and developed with ^{125}I-labeled protein A (*B*) as described.

ability to stimulate an antigen-specific H-2D^d-restricted T-cell hybridoma that responds to the antigen/MHC complex by growth inhibition (14). As shown in Fig. 5, SCβD^d_s molecules effectively presented the peptide antigen to this hybridoma in a dose-dependent fashion. On the order of 10- to 100-fold higher peptide concentrations were needed to provide T-cell stimulation with SCβD^d_s compared with the two-chain H-2D^d_s molecule. This result would be expected if, with tethered β_2m, the bound endogenous peptides were less able to dissociate and exchange with added antigenic peptides. It is possible that the single-chain molecules are not folded identically to the native molecule, and the T cells detect some subtle differences not detected by the conformationally sensitive antibody.

DISCUSSION

These data indicate the successful biosynthesis and secretion in stably transfected L cells of a T-cell stimulatory recombinant soluble single-chain class I MHC molecule SCβD^d_s. Correct folding is indicated by the presence of both the $\alpha_1\alpha_2$ and α_3 domain epitopes detected by ELISA (Fig. 2) and by immunoprecipitation (Fig. 3). The single-chain nature is confirmed by the presence of a 56-kDa band that reacts with antibodies to both H-2D^d (Fig. 3) and β_2m (Fig. 4). The functional ability of the single-chain molecule to react with a human immunodeficiency virus envelope glycoprotein 160-specific, H-2D^d-restricted T-cell hybridoma indicates that the antigenic peptide is bound in the same configuration as in the wild-type two-chain H-2D^d_s. The molecule described here, in contrast to that of Mottez *et al.* (4), has the opportunity to fold its rigid immunoglobulin-like β_2m domain first, providing a local high concentration of β_2m for the covalently linked H-2D^d_s and allowing β_2m to play its critical role in the formation of a structure available for peptide binding (13, 18, 21, 22). The thermostability and high mAb 34-5-8/mAb 34-2-12 ratio of SCβD^d_s (Fig. 2) suggest that self-peptides are effectively complexed to these molecules. Preliminary data indicate that endogenous peptides can be eluted from SCβD^d_s and that they seem to have the same sequence motif as the two-chain H-2D^d_s (M.M. and M.P.C., unpublished data). This result, in turn, indicates that a β_2m-free heavy-chain/peptide complex is not an obligate intermediate in the assembly of the heterotrimer and that SCβD^d_s, despite β_2m being tethered, allows sufficient flexibility for peptide binding.

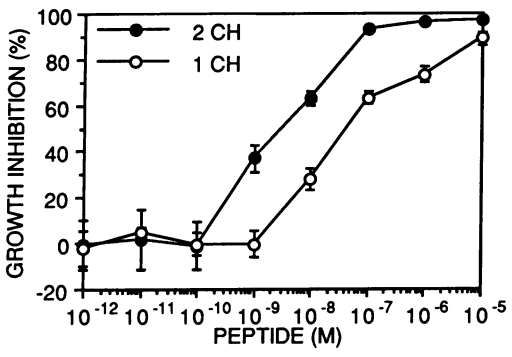

FIG. 5. Antigen presentation by SCβD^d_s, as measured by growth inhibition. Affinity-purified single-chain SCβD^d_s (○, 1 CH) or two-chain H-2D^d_s (●, 2 CH) molecules (33) were coated onto a microtiter plate and incubated with p18I10 at concentrations shown. After addition of B4.2.3 cells and incubation overnight, the cells were pulsed with [^{3}H]thymidine and harvested; radioactivity of incorporated label was then counted. Percent inhibition was calculated, and values plotted are means ± SEM of percent inhibition, calculated from triplicate wells. In absence of peptide, cpm were 170,000 for H-2D^d_s and 230,000 for SCβD^d_s. With a control H-2L^d-restricted peptide YPHFMPTNL (36) at 1 × 10^{-5} M, cpm were 210,000 for H-2D^d_s and 240,000 for SCβD^d_s.

We have demonstrated that synthesis, folding, and secretion of a single-chain class I MHC molecule with functional activity occurs in a mammalian cell line. Whether the synthesis of similar single-chain class I MHC molecules can be accomplished in other expression systems awaits determination; this work has been done for the two-chain class I molecules in baculovirus (37) and bacteria (38, 39). Further modification of the single-chain constructs could include the incorporation of toxin moieties and covalently linked antigenic peptide sequences. Such molecules offer potential for uniform peptide-loaded preparations for structural studies (39, 40) and might serve as components of vaccines, of toxin conjugates for killing autoimmune or malignant T cells, or of specifically labeled reagents for the imaging of specific T-cell clones.

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