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Immunology

Empty and peptide-containing conformers of class I major histocompatibility complex molecules expressed in *Drosophila melanogaster* cells

(antigen presentation/peptide binding/intracellular transport/cytotoxic T cell)

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ABSTRACT **Transfected *Drosophila melanogaster* cells can express large quantities of class I major histocompatibility complex molecules. Such molecules lack endogenous peptides because the *Drosophila* cells are devoid of proteins necessary for intracellular peptide loading. The empty molecules are efficiently expressed on the cell surface and can acquire extracellular peptides. The conformation and stability of empty murine class I molecules are determined by the source of β_2-microglobulin. All β_2-microglobulin-induced conformers of empty heavy chains seem to be unified in a common rigid conformation on peptide binding.**

CD8-expressing, cytotoxic T cells (CTLs) recognize and destroy virally infected cells. Their T-cell receptors interact with class I major histocompatibility complex (MHC) molecules containing viral peptides on the surface of the infected cells (1). T cells have to discriminate between class I molecules containing self peptides and those containing nonself peptides; thus it has been argued that T-cell receptors have been selected to discriminate between the differences in content in the peptide-binding groove (2–4). Bound peptides are believed to assume an extended conformation (2, 4), suggesting that the linear structure of the peptides may be the discriminating factor rather than an overall peptide–class I conformation. Conformational analyses of peptide–class I molecule complexes have been difficult, since procedures to obtain homogeneous class I molecules with a single bound peptide have been unavailable, although reconstitution of a denatured class I molecule has been reported (5). To alleviate this problem, we have expressed various mouse and human class I molecules in *Drosophila melanogaster* cells. Such cells can produce large quantities of empty class I molecules, which appear on the cell surface. In this communication we describe the characteristics of empty and homogeneous, peptide-containing molecules and demonstrate that these molecules can attain a variety of conformations.

MATERIALS AND METHODS

Antibodies, Peptides and cDNAs. Hybridomas Y3 (6), AF6 (6), 28.14.8s (7), 30.5.7 (7), B22.249 (7), W6/32 (8), 5F1 (9), and EH144 (6) were obtained from the American Type Culture Collection (Rockville, MD) or from the laboratory in which they were isolated. Antiserum 193 was generated by immunizing rabbits with a peptide encoding the carboxyl-terminal sequence of H-2K^b (LPDCKVMVHDPHSLA). Peptide epitopes from the following proteins were used: SIINFEKL (Ova) from ovalbumin (10); ASNENMETM, (NP) from influenza nucleoprotein (3); SERPQASGVYMGNL (LCMV Np) from lymphocytic choriomeningitis virus (LCMV) nucleoprotein (11); ILKEPVHGV (Pol) and FRIGCRHSR (Vpr) from human immunodeficiency virus polymerase and Vpr proteins (12). cDNAs encoding the mouse class I heavy chains K^b (13), L^d (11), and D^b (14), the human class I heavy chains A2.1 (15), A2.2 (15), B7 (15), and B27 (15), mouse β_2-microglobulin (mβ_2m) (16), human β_2m (hβ_2m) (17), and ovalbumin (18) were obtained from the laboratories in which they were isolated or by use of oligonucleotide primers and the polymerase chain reaction. pRMHa3 (19) and phshsneo (20) were kindly provided by M. McKeown (Salk Institute, San Diego).

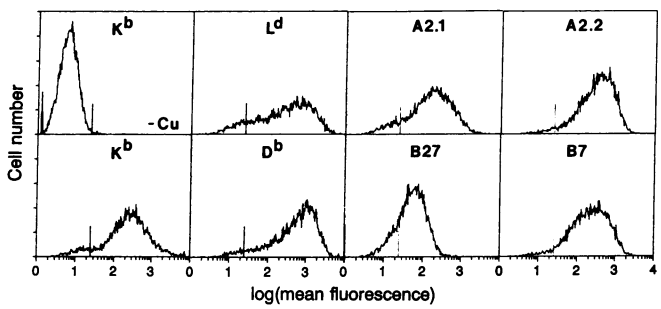

FIG. 1. Expression of class I MHC molecules by *Drosophila* cells. The stable *Drosophila* cell lines indicated were grown in medium containing 1 mM $CuSO_4$ for 24 hr prior to staining with anti-class I antibody and analysis by cytofluorometry. Antibodies Y3, 30.5.7, B22.249, and W6/32 were used to detect H-2K^b, H-2L^d, H-2D^b, and HLA, respectively. A marker indicates the position of the most fluorescent cells in negative controls (−Cu).

Cell Culture and Cytofluorometry. Schneider cells cultured in Schneider medium (GIBCO/BRL) supplemented with 10% fetal bovine serum were transfected by the calcium phosphate method with recombinant pRMHa3 DNA (24 μg) mixed with selection plasmid, phshsneo (1 μg) (21). After 48 hr, Geneticin (G418, 500 μg/ml; GIBCO/BRL) was added to the cultures. Four weeks later, populations of G418-resistant cells were converted to growth in the serum-free medium Excell 400 (JRH Biosciences). $CuSO_4$ (1 mM) was added to the medium and cells expressing class I molecules were sorted by using anti-class I antibodies and a Beckton Dickinson FACS 440.

Staining of cells for flow cytometry was carried out as follows. Cells induced with $CuSO_4$ (1 mM) for 24–48 hr were washed and resuspended in Excell 400 containing 0.02% NaN_3. Aliquots (10^6 cells) were either stained directly with antibody or stained after incubation in the presence of peptide (25 μM) or hβ_2m (Calbiochem) and then were incu-

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Abbreviations: β_2m, β_2-microglobulin; hβ_2m, human β_2m; mβ_2m, mouse β_2m; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex.

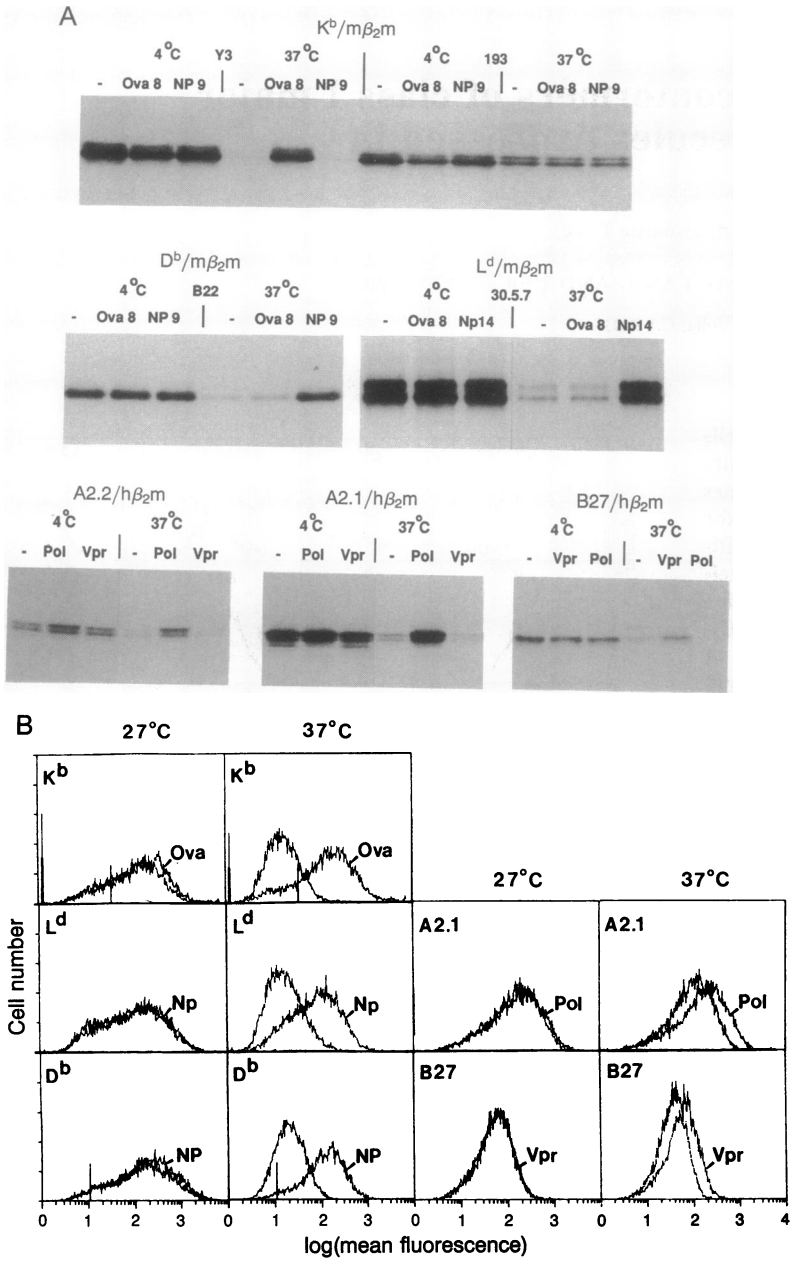

FIG. 2. Stabilization of empty class I MHC molecules by peptide. (*A*) Detergent lysates prepared from metabolically labeled cells were aliquoted and peptide was added as indicated (see *Materials and Methods* for peptide descriptions). After a 1-hr incubation at either 4°C or 37°C, class I molecules were immunoprecipitated with the appropriate antibodies (see Fig. 1) and analyzed by SDS/PAGE and autoradiography. (*B*) Prior to the analyses described in Fig. 1, peptide (25 μM) was added to cultures: Ova or Pol to K^b-, Ova or LCMV Np to L^d-, Ova or NP to D^b-, and either Pol or Vpr to HLA-expressing cultures. Cultures were then incubated at 27°C or 37°C for 2 hr. Thermostabilization of the surface expression of the class I molecules by the peptides indicated was observed, relative to the control peptide (unmarked).

bated for 2 hr at 27°C or 37°C. Binding of the primary antibody was detected with fluorescein-labeled goat anti-mouse IgG (Cappell) and a Beckton Dickinson FACS IV.

Osmotic Loading and CTL Assay. Osmotic loading of ovalbumin protein (3 mg/ml) or peptide (100 μM) was carried out as described (18), except that prior to peptide loading cells were incubated with 300 μM vesicular stomatitis virus peptide (RGYVYQGL) (3) for 1 hr. For CTL assays (10), 10^5 B3.1 T cells were cocultivated with 10^5 *Drosophila* or mouse 3T3 cells for 2 days, and the culture supernatant was assayed

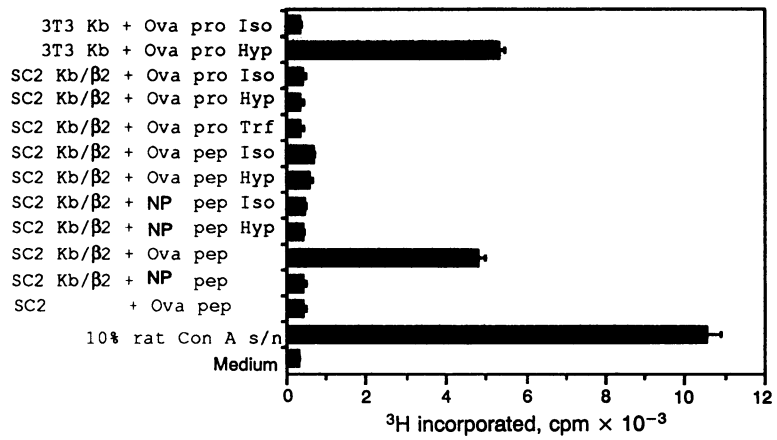

FIG. 3. Presentation of antigen by *Drosophila* cells expressing $K^b/m\beta_2m$. Mouse 3T3 fibroblasts (10) or *Drosophila* Schneider cells (SC2) expressing $K^b/m\beta_2m$ were incubated with ovalbumin protein (Ova pro), Ova peptide (pep) or NP peptide (3) in isotonic (Iso) or hypertonic (Hyp) medium. The treated cells and Schneider cells coexpressing $K^b/m\beta_2m$ and ovalbumin protein from a transfected cDNA (Trf) were cocultured with B3.1 cells (10). Levels of interleukin 2 secreted by the B3.1 cells were determined by incorporation of [^{3}H]thymidine into CTLL-2 cells. Medium supplemented with 10% supernatant (s/n) from Con A-stimulated rat peripheral blood lymphocytes (10) and medium alone were used as positive and negative controls, respectively. Untransfected Schneider cells incubated with Ova peptide were used as controls for K^b-dependent presentation of Ova peptide.

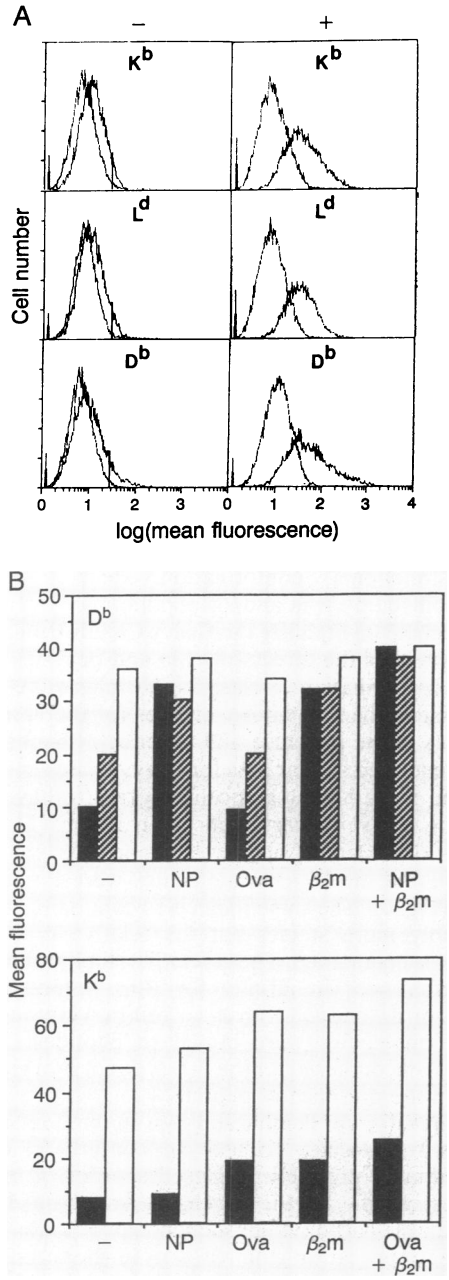

FIG. 4. Mouse class I heavy chains are transported to the cell surface in the absence of β_2m. (*A*) Drosophila cells expressing class I heavy chains alone grown in Excell medium with (+) or without (–) 10% fetal bovine serum were stained as described in Fig. 1. Flow cytometry profiles of cells grown and stained under the same conditions but without $CuSO_4$ are shown overlaid. (*B*) Analysis of class I expression as in *A*, except that cells were cultured in medium containing NP or Ova peptide (25 μM), or hβ_2m (3 μg/ml), or peptide and hβ_2m, or with no additions (–). Expression of D^b was detected with B22.249 (filled bars), 28-14-8S (hatched bars), or a polyclonal anti-H-2 rabbit antiserum, K270 (26) (open bars). Expression of K^b was detected with Y3 (filled bars) or K270 (open bars). Mean linear fluorescence values obtained are shown plotted against the overnight incubation conditions.

for interleukin 2 activity on CTLL-2 cells. The B3.1 T-cell hybridoma was obtained from F. Carbone (10).

Metabolic Labeling. Cells were labeled in methionine-free Grace's medium (GIBCO/BRL) containing [^{35}S]methionine/[^{35}S]cysteine (Tran^{35}S-label, 0.1 μCi/ml, NEN; 1 μCi = 37 kBq). After labeling, cells were lysed in ice-cold phosphate-buffered saline/1% (vol/vol) Triton X-100 with protease inhibitors and precleared by centrifugation. After 1 hr at 4°C in the presence of peptide (25 μM), aliquots were incubated at various temperatures (4–45°C) for 1 hr. MHC class I molecules were isolated by immunoprecipitation and analyzed by SDS/10–15% PAGE.

RESULTS

Transfected *Drosophila* Cells Express Empty Class I Molecules. *D. melanogaster* lacks a conventional immune system, and MHC genes have not been identified in this species. Auxiliary proteins required for loading peptide onto class I molecules in mammalian cells are encoded in the MHC region (22); it therefore seemed likely that *Drosophila* cells transfected with cDNAs encoding the class I subunits would express class I molecules free of peptide (7, 23, 24). cDNAs encoding various mouse and human class I subunits were cloned downstream of the metallothionein promoter in the *Drosophila* expression vector pRMHa3 (19). Stable cell lines transfected with the recombinant plasmids encoding heavy chain and β_2m were established. Flow cytometry analyses with anti-class I antibodies (Fig. 1) showed that surface expression of the various MHC class I molecules in these lines was copper-dependent. Since binding by these antibodies requires β_2m to be associated with the heavy chain (6–8), we obviously detected expression of the heterodimer. To determine whether the expressed molecules were free of peptide, we took advantage of the fact that empty class I molecules (7) are more thermolabile than peptide-containing molecules (23, 24). To this end we immunoprecipitated class I molecules from the *Drosophila* cells after exposing lysates for 1 hr to either 4°C or 37°C. Prior to these incubations, peptides known to bind to the various class I molecules were added to the lysates. SDS/PAGE analyses of the immunoprecipitated class I molecules (Fig. 2*A*) indicated that at 4°C all the class I molecules were stable. The class I molecules run as a doublet on SDS/PAGE due to trimming of the N-linked carbohydrates in the Golgi. After incubation at 37°C, few if any class I molecules were immunoprecipitated unless peptide with affinity for the class I molecules had been added or antibodies (antiserum 193) that do not rely on class I conformation were used (Fig. 2*A*). The temperature-sensitive nature of the expressed class I molecules was confirmed by flow cytometry (Fig. 2*B*). At 37°C class I molecules vanished from the cell surface unless they had been exposed to peptide that binds (Fig. 2*B*). These results demonstrate that human and mouse MHC class I molecules expressed in *Drosophila* cells display all the hallmarks of empty molecules (23, 24).

Class I Molecules on *Drosophila* Cells Can Present Exogenous but Not Endogenous Antigens to T Cells. A T-cell hybridoma reactive with K^b molecules containing a peptide (Ova) from ovalbumin (10, 18) was used to determine whether K^b/β_2m-expressing *Drosophila* cells could process and present antigen. Prior to the assay the cytoplasm of the *Drosophila* cells had obtained intact ovalbumin or Ova peptide by osmotic loading (18). This procedure resulted in efficient antigen presentation by 3T3 cells expressing K^b molecules (Fig. 3) but not by K^b/β_2m *Drosophila* cells. The inability of K^b/β_2m *Drosophila* cells to stimulate T cells was not due to inefficient loading, since fluorescein-labeled dextran was readily introduced into the cytoplasm by this procedure, nor was it due to the T cells being incompatible with *Drosophila* cells, as K^b/β_2m *Drosophila* cells cultured in the presence of Ova peptide stimulated the T cells. Furthermore, *Drosophila* cells coexpressing K^b/β_2m and the ovalbumin protein did not stimulate the T cells (Fig. 3). These data suggest that although *Drosophila* cells are unable to present intracellular antigen, they efficiently assemble functional MHC class I molecules and express them on their surface.

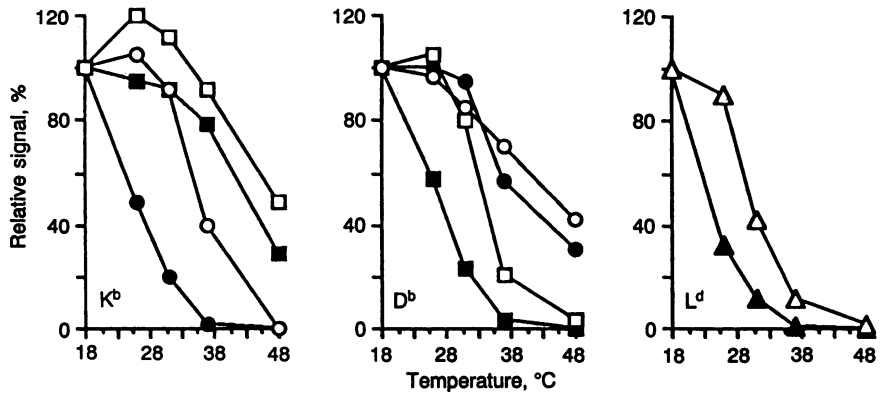

FIG. 5. Peptide and β_2m stabilization of class I molecules. Aliquots of detergent lysates of metabolically labeled *Drosophila* cells were incubated for 1 hr at 18, 24, 31, 37, and 48°C, and class I molecules were immunoprecipitated and analyzed as in Fig. 2. Amount of heavy chain immunoprecipitated at each temperature was quantitated with a densitometer and is expressed as a percentage of the amount of heavy chain immunoprecipitated at 18°C. Shown are temperature profiles of the indicated H-2 molecules coexpressed with mβ_2m (filled symbols) or hβ_2m (open symbols) and the effect of adding Ova (squares) or NP (circles) peptide to the lysates.

β_2m and Peptides Interact with a Distinct Conformation of Class I Heavy Chains. The prevailing view is that β_2m is essential for the intracellular transport of class I molecules (25), a view contradicted by the observation that D^b heavy chains are expressed at the cell surface in a β_2m-deficient cell line, although in a distorted conformation (25). To address this issue we established *Drosophila* cell lines stably expressing heavy chains alone. Flow cytometry showed that K^b, L^d, and D^b all occurred at the cell surface (Fig. 4*A*) and in a conformation recognized by antibodies reacting with "β_2m-dependent" epitopes (6, 7). These data suggested that β_2m present in the medium had complemented the heavy chains at the cell surface, a notion confirmed by flow cytometry analyses of cells grown in serum-free medium (Fig. 4*A*). Addition of β_2m to the serum-free medium 1 hr before the analyses resulted in only a small increase in the binding of β_2m-dependent anti-class I antibodies (data not shown), indicating that free heavy chains had reached the cell surface in a conformation that could bind β_2m. However, if this protein was not in the medium, the heavy chains underwent an irreversible conformational change. These findings prompted us to ask whether peptides with affinity for class I molecules might also be able to stabilize free heavy chains. When peptides known to bind to K^b (Ova) or D^b (NP) were added to the serum-free medium, surface expression of the heavy chains increased (Fig. 4*B*). However, when both peptide and β_2m were present in the medium, only a fraction of the K^b molecules were maintained in a conformation recognized by the Y3 antibody. Binding of peptide to K^b heavy chains was also analyzed in lysates. Immunoprecipitations with antibodies that recognized a β_2m-dependent conformation of K^b could not precipitate free heavy chains unless they had been exposed to Ova peptide (see Fig. 6). This information, in conjunction with similar data for free D^b chains (21), supports the view that free heavy chains can attain a "functional" conformation, which is short-lived, unless stabilized by β_2m and/or peptide.

hβ_2m and mβ_2m Stabilize the Heavy Chains Differently. To examine whether the effects of β_2m and peptides on stabilizing the conformation of class I molecules were separate, we established thermostability profiles for the various class I molecules in the presence and absence of bound peptide. We also generated similar data for heavy chains coexpressed with hβ_2m, since hβ_2m has been reported to have a higher affinity for mouse heavy chains than mβ_2m (27). L^d/β_2m molecules lost reactivity at a lower temperature than K^b/β_2m molecules, and the D^b/β_2m molecules were the most heat-resistant (Fig. 5). The same molecules containing hβ_2m were considerably more stable, but the relative rank order of stability was maintained. These relative stabilities most likely reflect differences in affinity for β_2m. Interestingly, the stabilities of K^b and D^b with h- or mβ_2m were quite similar when the molecules were bound to peptides. Thus, the differences encountered for the two types of β_2m were largely negated in the presence of peptide.

Different Effects of Peptide and β_2m on the Conformation of the Class I Molecule. To analyze conformers of class I molecules more precisely, we used a series of monoclonal antibodies specific for the K^b chain. Antiserum 193, which reacts with the carboxyl-tail sequence of K^b, was also used, with the expectation that it would be conformation-insensitive. However, fewer free K^b chains were immunoprecipitated with the 193 antiserum at 30°C than at 4°C, regardless of whether a peptide known to bind to K^b had been added (Fig. 6). This indicated that the cytoplasmic tail of K^b displayed a temperature-sensitive conformation, a view supported by analysis of $K^b/m\beta_2$m (Figs. 2 and 6). Binding of the Ova peptide to $K^b/m\beta_2$m also seemed to have a slight effect inasmuch as its presence reduced the amount of immunoprecipitated material. This effect was considerably more pro-

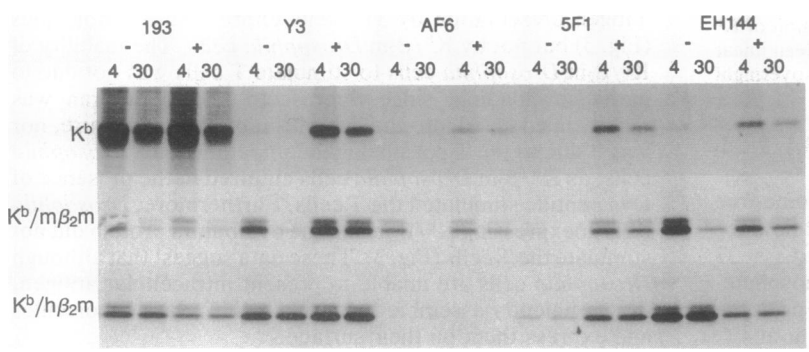

FIG. 6. Conformation of K^b heavy chain. Ova (+) or NP (−) peptide was added to Triton X-100 lysates prepared from labeled *Drosophila* cells expressing K^b, $K^b/m\beta_2$m, or $K^b/h\beta_2$m. After an incubation at 4°C or 30°C for 1 hr, K^b heavy chain was immunoprecipitated from the different conditions by using the antibodies indicated, prior to analysis by SDS/PAGE and autoradiography.

nounced for the $K^b/h\beta_2m$ molecules. Binding of the Ova peptide resulted in a molecule that was more poorly recognized by the 193 antiserum. These data suggest that the cytoplasmic tail of the K^b molecule can occur in different conformations, which are influenced both by peptide and by β_2m binding.

Antibody Y3 [α2-specific (6)] recognized both empty and peptide-containing K^b/β_2m molecules, although free K^b chains were recognized poorly unless they contained peptide. Thus, Y3 antibodies recognized a conformation-sensitive epitope that could be generated either by β_2m (human or mouse) or by peptide. Another antibody, AF6 (6), displayed a qualitative reactivity pattern similar to that of Y3 when recognizing free K^b chains and $K^b/m\beta_2m$ molecules. However, it completely failed to react with $K^b/h\beta_2m$ molecules (28). The AF6 data confirmed that the greater thermal stability of the $K^b/h\beta_2m$ molecules was accompanied by a conformation that distinguished it from $K^b/m\beta_2m$ molecules.

Recognition of K^b conformers by Y3 and AF6 antibodies was primarily affected by the presence of β_2m, whereas antibodies 5F1 [α2-specific (6)] and EH144 [α1-specific (6)] seemed to recognize primarily peptide-dependent conformations. Thus, 5F1 efficiently precipitated all forms of K^b molecules, provided the Ova peptide was present, but reacted poorly with molecules lacking the bound peptide. The reactivity pattern of EH144 was not as clear-cut, but empty molecules containing β_2m were better recognized than K^b molecules containing peptide (Fig. 6). However, free K^b chains in the presence of the Ova peptide were more reactive than in the absence of the peptide. These analyses suggested that class I molecules can attain a variety of conformations that can be recognized by antibodies. It is clear that the source of β_2m and an occupied peptide-binding groove affect these conformations. It can further be concluded that the conformational effects of β_2m and peptide on the K^b chain are largely independent, although some overlap seemed to exist.

DISCUSSION

This paper demonstrates that transfected *Drosophila* cells can serve as an excellent source of empty class I molecules. Although such cells lack the auxiliary proteins necessary for peptide loading, they efficiently assemble class I molecules and transport them to the cell surface. Empty class I molecules are thermolabile at 37°C (23, 24), but since *Drosophila* cells are normally grown at 27°C the empty heterodimers accumulate to high levels. This is in marked contrast to the failure to express significant amounts of assembled, empty class I molecules in *Spodoptera frugiperda* cells (29).

The present data show that empty and peptide-containing class I molecules attain different conformations. The conformation of the empty molecules was obviously affected by the source of β_2m, but once a peptide was bound the different conformational effects of $m\beta_2m$ and $h\beta_2m$ were less apparent. This suggests that the epitopes of the class I-β_2m molecules containing a peptide might attain a unified or "end" conformation, which is largely independent of the source of β_2m.

The finding that peptide-containing K^b heavy chains are more stable when associated with $h\beta_2m$ rather than $m\beta_2m$ suggests that β_2m and peptide have different but complementary conformational effects. Whether different peptides, like different β_2m chains, can induce discrete conformations recognizable by antibodies requires further investigation.

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