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Immunology

Reassociation with β_2-microglobulin is necessary for K^b class I major histocompatibility complex binding of exogenous peptides

(antigen presentation/T lymphocyte/cytotoxic T lymphocyte)

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ABSTRACT **T lymphocytes recognize endogenously produced antigenic peptides in association with major histocompatibility complex (MHC)-encoded molecules. Peptides from the extracellular fluid can be displayed in association with class I and class II MHC molecules. Here we report that mature K^b class I MHC molecules bind peptides upon dissociation and reassociation of their light chain. Intact K^b heterodimers, unlike class II MHC molecules, are relatively unreceptive to binding peptides. This property may maintain segregation of class I and class II MHC-restricted peptides and has implications for the use of peptides as vaccines.**

Cytotoxic T lymphocytes (CTLs) and T helper cells recognize processed antigenic peptides in association with class I and class II major histocompatibility complex (MHC) gene products, respectively, on the surface of antigen-presenting cells (APCs) (1). There are differences in the specificity of CTL versus T helper cell responses, the existence of which reflects the distinct spectra of antigens (Ags) that are presented in association with the two classes of MHC molecules. Ags that are acquired from the extracellular fluid are presented in association with class II MHC molecules, whereas those that are endogenously synthesized are presented in association with class I MHC molecules (2).

Despite these differences, there are striking similarities in the manner in which both sets of MHC molecules function. Antigenic peptides are bound and displayed in specific sites formed by the polymorphic domains of both classes of MHC molecules (3–5). Furthermore, class I and class II MHC molecule–Ag complexes can be formed by incubating APCs with exogenous peptides (6, 7). Moreover, there is a remarkable similarity in the peptide motifs that bind to these two sets of MHC-encoded molecules (8); in some cases a single peptide will bind to, and be presented by, class I and class II MHC molecules (9). Despite these similarities in function, in this report we described marked operational differences in the manner in which peptides bind to class I versus class II MHC molecules.

MATERIALS AND METHODS

Reagents. Chicken ovalbumin (OVA) and human β_2-microglobulin (β_2m) were purchased from Sigma. OVA was treated with trypsin (t-OVA) and cyanogen bromide (CNBr-OVA) as described (6). The $OVA_{258-276}$ peptide was synthesized at the Dana Farber Cancer Institute.

Cell Lines and Monoclonal Antibodies (mAbs). The following T-T hybridomas were used: RF33.70 [C57BL/6 (anti-OVA-plus-K^b) hybridized with BW.CD8.7] (10), 8DO51.15, and DO.11.10 [BALB/c (anti-OVA-plus-IA^d), hybridized with BW5147], kindly provided by J. Kappler and P. Marrack (National Jewish Center, Denver, CO) (11, 12). The LB27.4 cell line is an Ia-positive, H-$2^{b\times d}$ B-B hybridoma (13). The EL4 cell line is a T-cell lymphoma of C57BL/6 (H-2^b) origin. mAb-containing culture supernatants were prepared from the hybridoma cell lines M1/42 (anti-murine MHC class I molecules) (14), BBM.1 (anti-human β_2m) (15), and 28.14.8S (anti-D^b) (16).

Cell Culture. LB27.4 or EL4 APCs were antigen exposed as described (17) in the presence or absence of serum or β_2m. Osmotic lysis of pinosomes was done as described (18, 19) and is referred to as "Ag loading." Incubations at low serum concentrations (0–1%) were done in Optimem medium (GIBCO) supplemented with 1% Nutridoma SP (Boehringer Mannheim). All other incubations and cultures were done in RPMI 1640 medium, supplemented as described (17). APCs were fixed with paraformaldehyde or glutaraldehyde as described (6, 20). Hybridoma cell cultures and interleukin 2 assays were done as described (17). The stimulation of OVA-specific CTLs and ^{51}Cr release assays were done as described (10).

RESULTS

Kinetics of Peptide–MHC Association. To examine the kinetics of peptide association with class I versus class II MHC molecules, APCs were incubated with exogenous t-OVA for varying times and were then fixed. The presence of peptide–MHC molecule complexes on the pulsed APCs was then detected with class I or class II MHC-restricted T-T hybridomas. The association of t-OVA with IA^d molecules on LB27.4 APCs occurs rapidly (Fig. 1*C*), even at low peptide concentrations and when the APCs are fixed (Fig. 1*D*), which indicates that there are Ia molecules on the cell surface that are highly receptive to exogenous peptides. In contrast, the association of t-OVA with class I MHC molecules on the same APCs requires extended incubation in relatively high concentrations of peptide (Fig. 1*B*). The slow association between the exogenous OVA peptide and K^b molecules is observed with B APCs (Fig. 1*B*) and T APCs (Fig. 1*A*). The EL4 APCs are more active than LB27.4, which may reflect the higher level of expression of K^b on the former (unpublished data). Furthermore, this same phenomenon is also observed with CNBr-OVA, which is ≈100-fold more active than t-OVA (unpublished data, also see Fig. 5). Therefore, this slow association does not appear to be unique to a single and/or "weak" peptide. Differences in the sensitivity of the class I and class II MHC-restricted hybridomas do not account for these marked differences (unpublished data). Taken together, our results demonstrate that OVA peptides

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Abbreviations: Ag, antigen; APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; mAb, monoclonal antibody; NMS, normal mouse serum; OVA, ovalbumin; t-OVA, trypsin-treated OVA; CNBr-OVA, CNBr-treated OVA; β_2m, β_2-microglobulin.

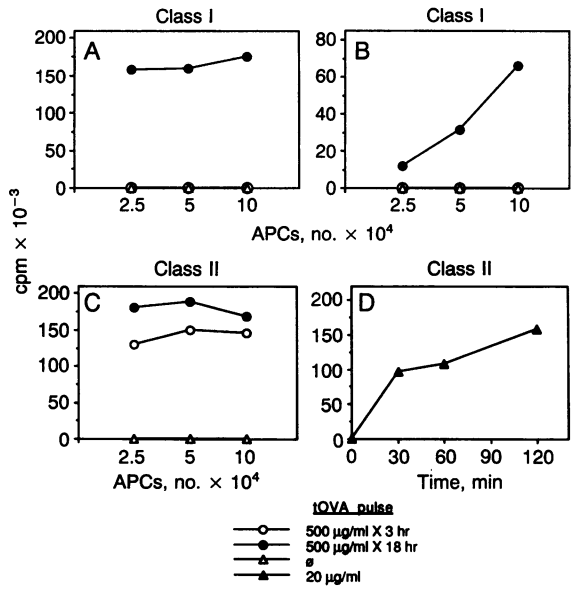

FIG. 1. Kinetics of the association of exogenous peptides with class I and class II MHC molecules. (*A–C*) APCs were incubated with t-OVA (500 μg/ml) in RPMI 1640 (10% fetal calf serum) medium for 3 or 18 hr at 37°C and were subsequently washed and fixed with paraformaldehyde. The indicated numbers of Ag-exposed and fixed APCs were added to cultures with T-T hybridomas. After 18 hr of incubation at 37°C, 100 μl of supernatant was removed from duplicate cultures and assayed for interleukin 2 content. (*A*) RF33.70 (anti-OVA-plus-K^b) cells with EL4 APCs. (*B*) RF33.70 cells with LB27.4 APCs. (*C*) 8DO51.15 (anti-OVA-plus-IA^d) cells with LB27.4 APCs (the Ag-exposed APCs in *B* and *C* are from the same group). (*D*) LB27.4 APCs were fixed with paraformaldehyde and incubated with t-OVA (20 μg/ml) for the indicated length of time at 37°C. The fixed and Ag-exposed APCs (5×10^4) were added to cultures with DO.11.10 (anti-OVA-plus-IA^d) cells.

associate much more readily with class II MHC molecules than K^b class I MHC molecules on the surface of APCs.

Endogenous Peptide–Class I MHC Association. To examine the kinetics of formation of peptide–MHC complexes arising from endogenously processed Ag, OVA was introduced into the cytoplasm of APCs by means of osmotic lysis of pinosomes (19) and the APCs were fixed at varying times thereafter. The appearance of OVA–K^b complexes on the APC surface is detectable after 15–30 min and is near maximal by 1 hr after Ag loading (Fig. 2*A*). This rapid appearance of Ag–class I MHC complexes is observed even under limiting conditions of Ag (unpublished data). These results indicate that peptide–class I MHC complexes can form rapidly, which resembles the behavior of class II MHC molecules (Fig. 2*B*) more closely than that of the mature class I MHC molecules on the APC surface (Fig. 1).

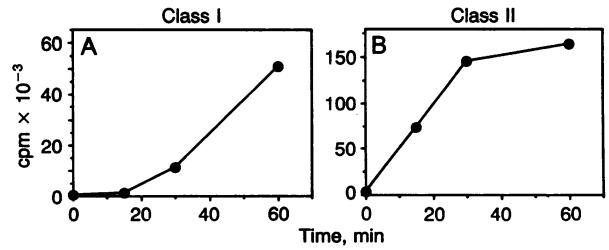

FIG. 2. Endogenously processed Ag rapidly associates with class I and class II MHC molecules. LB27.4 APCs were incubated with OVA (30 mg/ml) in hypertonic buffer for 10 min at 37°C; this was followed by a 3-min incubation in hypotonic buffer as described (18, 19) and washing at 4°C. The Ag-loaded APCs were incubated at 37°C for the indicated length of time and were then fixed with paraformaldehyde. Microcultures were prepared as described in the legend to Fig. 1 and contained RF33.70 (anti-OVA-plus-K^b) cells and LB27.4 cells (10^5) (*A*) and DO.11.10 (anti-OVA-plus-IA^d) cells and LB27.4 cells (1.25×10^4) (*B*).

It is possible that the difference in the kinetics of Ag association for intracellular versus extracellular class I MHC molecules reflects a difference in the specific peptides that are being offered. However, when we examined different related OVA peptides, we found no evidence for such an effect (see above).

β_2m and Peptide Association. Further studies demonstrated additional differences between the formation of peptide–K^b class I MHC complexes intracellularly and extracellularly as well as between peptide pulsing of class I and class II MHC molecules on the cell surface. The formation of peptide–K^b class I MHC complexes, which occurs upon the extended incubation of APCs with exogenous peptide, is markedly influenced by the exogenous serum concentration. As shown in Fig. 3*A*, t-OVA associates with class I MHC in the presence of 10%, but not 1%, serum. The critical variable in these experiments is the concentration of serum, rather than the species of origin of the serum, although the heterologous fetal serum is more active (Fig. 3*A*). Moreover, when these APCs are loaded with native OVA, the generation of OVA–class I MHC complexes from endogenously processed Ag and intracellular class I MHC molecules is not affected by exogenous serum (Fig. 3*C*). In contrast to class I, the association of peptides with class II MHC molecules on the APC surface is not dependent on exogenous serum (Fig. 3*B*). These latter observations demonstrate the functional competence of the APCs cultured in low serum concentrations. Therefore, these results indicate that a component found in serum may be necessary or facilitate peptide association uniquely with class I MHC molecules on the APC surface.

Serum contains free β_2m that is capable of associating with the heavy chain of class I MHC heterodimers on the plasma membrane (21). This association can occur with exogenous, xenogeneic β_2m (22). We therefore tested whether exogenous

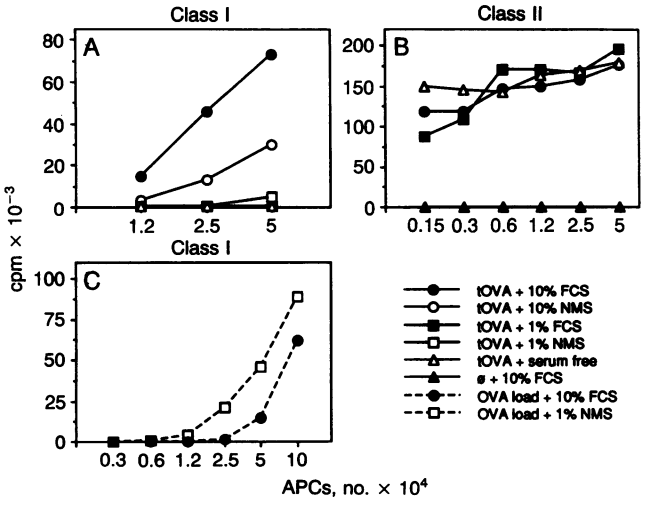

FIG. 3. The association of exogenous peptide with class I MHC molecules is dependent on serum. The LB27.4 APCs in this experiment were adapted to grow in serum-free medium. LB27.4 APCs were incubated for 18 hr at 37°C with 400 μg of t-OVA per ml (*A*) or 100 μg of t-OVA per ml (*B*). (*C*) APCs were loaded with OVA (20 mg/ml) by means of osmotic lysis of pinosomes and were incubated for 1 hr at 37°C. The indicated concentration of normal mouse serum (NMS) or fetal calf serum (FCS) was present during the peptide pulse or in the incubation following the OVA loading. The APCs were subsequently fixed with paraformaldehyde. Microcultures were prepared as described in the legend to Fig. 1 and contained RF33.70 (anti-OVA-plus-K^b) cells with t-OVA-exposed APCs (*A*), DO.11.10 (anti-OVA-plus-IA^d) cells with t-OVA-exposed APCs (*B*), and RF33.70 cells with OVA-loaded APCs (*C*).

β_2m affected the association of the OVA peptides with K^b molecules. EL4 or LB27.4 APCs were pulsed with t-OVA in medium containing low amounts of serum and in the presence or absence of purified human β_2m and were then washed and fixed. In the presence of exogenous β_2m, there is extremely effective pulsing of class I MHC molecules on both APCs (Fig. 4). Under these conditions, the association of peptide is rapid and can be detected after 30–60 min (Fig. 4*C*). The optimal concentration of exogenous β_2m is >10 μg/ml (Fig. 4*B*). The sensitization of the APC is a function of the concentrations of peptide and β_2m (Fig. 4*B*). As this experiment would predict, the formation of OVA–class I MHC complexes can occur in a 2- to 3-hr incubation period in the presence of low concentrations of β_2m, such as are present in 10% serum, provided that a sufficiently high concentration of peptide is present (unpublished data; see also Fig. 5). The species origin of the β_2m does not appear to be critical (Figs. 3*A* and 4). The presence of the exogenous, purified β_2m does not affect the association of OVA peptides with class II MHC molecules (unpublished data).

We next examined whether these results were influenced by the nature of the T-cell effector population, the assay system, the particular peptide preparation, and/or the concentration of the peptide. For this purpose, we first examined the presentation of peptide to polyclonal OVA-specific CTLs in a ^{51}Cr release assay. Moore *et al.* (19) have previously shown that such polyclonal CTLs recognize CNBr-cleaved OVA peptides, and specifically an epitope within $OVA_{258-276}$, in association with K^b. In this system, the rapid (2 hr) sensitization of EL4 target cells with 8 μM $OVA_{258-276}$ peptide under serum-free conditions requires the presence of exogenous β_2m (Fig. 5*A*). Identical results are obtained in assays with T-T hybridomas and the $OVA_{258-276}$ peptide (Fig. 5*B*) or CNBr-OVA (unpublished data; see also Fig. 5*C*). The CNBr-OVA is however ≈100-fold more potent than t-OVA in the latter system (Fig. 5*D* and unpublished data). We conclude that the same results are obtained for several related OVA peptides in conventional or hybridoma assay systems.

In contrast to the above results, we observed that at high concentration (60 μM $OVA_{258-276}$ or 100 μg of CNBr-OVA per ml) peptide could associate with K^b molecules on EL4 cells during a 2-hr incubation in the apparent absence of exogenous β_2m (Fig. 5 *A* and *C*). It was recently suggested that exogenous peptides present at high concentrations can enter cells and associate with newly synthesized class I MHC molecules in the endoplasmic reticulum (23). To determine whether this pathway might account for the sensitization with peptide in the absence of exogenous β_2m, we assessed the effects of treating the APCs with metabolic inhibitors. Under serum-free conditions, the association of CNBr-OVA with K^b molecules on EL4 cells is virtually completely inhibited by azide (Fig. 5*C*) or ricin (unpublished data). In contrast, these inhibitors do not prevent the association of peptide with K^b molecules in the presence of exogenous β_2m (Fig. 5*C* and unpublished data). Similarly, the EL4 cells that are treated with inhibitors and then fixed are not impaired in their ability to subsequently present peptide added to an 18-hr hybridoma culture in the presence of serum (Fig. 5*D*). These latter results suggest that the metabolic inhibitors have not simply damaged the APC. Taken together these results illustrate that there can be a pathway of peptide association with class I molecules that is dependent on metabolic energy and *de novo* protein synthesis.

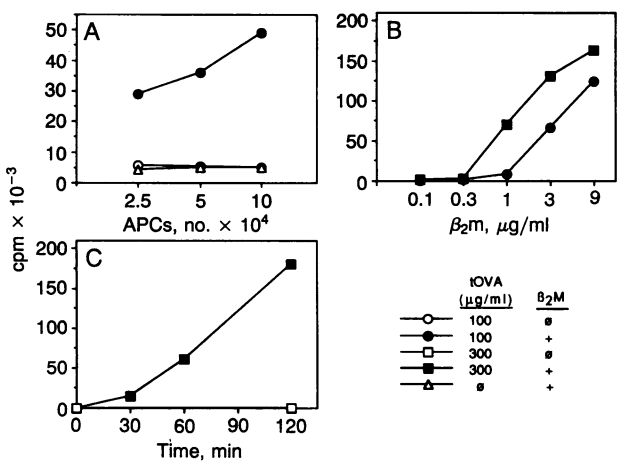

FIG. 4. Peptide rapidly associates with class I MHC molecules in the presence of exogenous purified β_2m. APCs were incubated with or without t-OVA (100 or 300 μg/ml) in medium containing 1% NMS in the presence or absence of human β_2m (purity >95%). After 2–3 hr of incubation at 37°C, the APCs were washed and fixed with paraformaldehyde. Microcultures were prepared as described in the legend to Fig. 1 and contained RF33.70 (anti-OVA-plus-K^b) cells and LB27.4 APCs exposed to Ag with or without β_2m (2.5 μg/ml) for 3 hr (*A*), RF33.70 cells and EL4 APCs (10^5) exposed to Ag with or without β_2m (0.1–9 μg/ml) for 2 hr (*B*), and RF33.70 cells and EL4 APCs (10^5) exposed to Ag with or without β_2m (9 μg/ml) for 0.5–2 hr (*C*).

We next asked whether preincubation of APCs with exogenous β_2m would "prepare" class I MHC molecules to associate with peptide or whether it was necessary for the β_2m to be present at the time of peptide association. APCs that have been preincubated with purified human β_2m, washed, and then incubated with peptide for 3 hr do not present t-OVA in association with class I MHC molecules (Fig. 5). The association of t-OVA with K^b requires that the exogenous β_2m be present at the time of peptide pulsing (Fig. 5). These results suggest that exogenous OVA peptides associate with class I MHC molecules during the reassociation of β_2m and not by the Ag associating with preexisting, "free" class I MHC, peptide binding sites, on the cell surface.

If peptide association does indeed occur in these experimental conditions during the reassociation of β_2m, then peptides should only be present in class I MHC molecules that have a light chain of exogenous origin. To test this hypothesis, APCs were pulsed with t-OVA in the presence of NMS (as a source of exogenous murine β_2m) or exogenous human β_2m. The APCs were then incubated with mAbs that are specific for either mouse–mouse or mouse–human class I MHC heterodimers. As a control comparison, the same APCs were incubated with the 28.14.8S mAb, whose binding is not dependent on β_2m (24). T-cell responses to APCs pulsed with t-OVA and mouse β_2m are inhibited by the M1/42 mAb, which is specific for murine–murine class I MHC heterodimers (22), and are unaffected by the BBM.1 mAb, which is specific for human β_2m (Fig. 6). In contrast, a reciprocal pattern of inhibition is observed when the same APCs have been pulsed with t-OVA in the presence of exogenous human β_2m (Fig. 6). The inhibition that is observed with the anti-human β_2m mAb is only partial; however, this does not reflect the presence of peptides bound to murine–murine class I MHC heterodimers, since the M1/42 mAb does not cause inhibition. The partial effect of the BBM.1 mAb could reflect the location of the bound mAb (distant from the peptide/T-cell receptor interaction sites), elution of the bound mAb, and/or exchange with bovine β_2m. Taken together, these results indicate that the association of OVA peptides with K^b molecules on the cell surface requires the disruption and reformation of the class I MHC heterodimer.

DISCUSSION

Our results provide several observations that are relevant to understanding how peptides associate with class I MHC

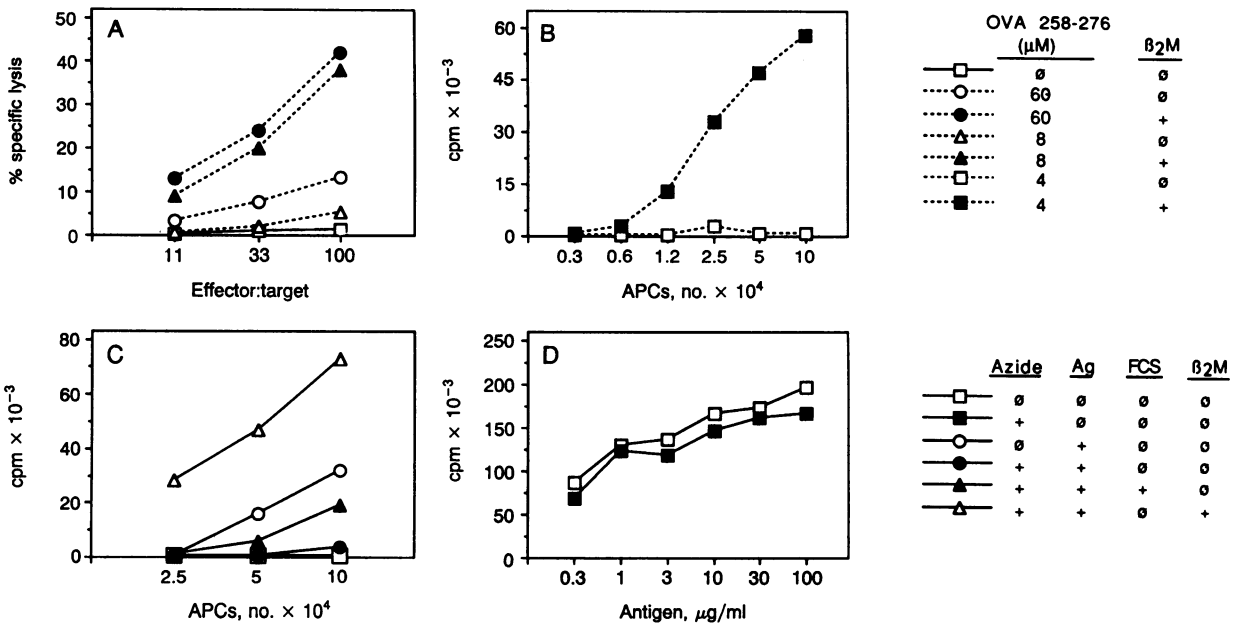

FIG. 5. Energy-dependent and -independent pathways of peptide association with K^b molecules. APCs were either untreated, incubated with ^{51}Cr for 18 hr at 37°C, or incubated for 1 hr at 37°C with azide (45 mM). The APCs were subsequently incubated with or without either $OVA_{258-276}$ (4–60 μM) or CNBr-OVA (100 μg/ml) and in the presence or absence of either human β_2m (10 μg/ml) or fetal calf serum (FCS; 10%) in Optimem medium. In groups with azide, the drug was continuously present during the Ag exposure. After 2 hr of incubation with Ag at 37°C, the APCs were washed and in some cases (*B–D*) fixed with paraformaldehyde. (*A*) ^{51}Cr-labeled EL4 APCs that were exposed to $OVA_{258-276}$ with or without human β_2m were incubated with polyclonal OVA-specific CTLs for 4 hr at 37°C, and the specific release of ^{51}Cr was measured. (*B–D*) Microcultures were prepared as described in the legend to Fig. 1 and contained RF33.70 (anti-OVA-plus K^b) cells and EL4 APCs exposed to $OVA_{258-276}$ with or without human β_2m (*B*), RF33.70 cells and azide or untreated EL4 APCs exposed to CNBr-OVA with or without human β_2m or FCS (*C*), and RF33.70 cells and azide or untreated EL4 APCs (5×10^4) with CNBr-OVA added to the hybridoma culture in 10% FCS (*D*).

molecules. Free, exogenous β_2m markedly facilitates the association of OVA peptides with the K^b molecule on the surface of APCs. These peptides are found primarily on class I MHC molecules whose light chain is derived from exogenous β_2m. It is also necessary that the exogenous β_2m be present at the time of peptide association. These observations strongly suggest that peptide is associating with K^b during dissociation or reassociation of the class I MHC heterodimer. It is possible that peptide is binding to heterodimers that are undergoing exchange of their light chain or to already dissociated class I MHC molecules.

Our results imply that the peptide binding site of previously assembled class I MHC molecules is affected by β_2m dissociation/reassociation. This effect is likely to be indirect because the amino acid residues of β_2m are not present in the putative peptide association site (4). β_2m does contact the $\alpha 2$ and $\alpha 3$ domains of the class I MHC heavy chain, which form the peptide binding site (4). There is evidence that β_2m may affect the conformation of the class I MHC heavy chain (22, 24).

Recently peptides were found to induce the association of class I heavy and light chains in a mutant cell line that has a defect in the assembly of the class I MHC heterodimer (23). Therefore the binding of endogenous peptides may be required for and/or occur during the biosynthetic assembly of the class I MHC molecule. Further study will be necessary to determine whether the mechanism of association for peptides with newly synthesized and mature extracellular K^b class I MHC molecules is similar.

Our results provide several observations that may be relevant to understanding class I and class II MHC function. Available data suggest that class I and class II MHC molecules have similar peptide binding sites and bind similar peptides (4, 5, 8, 9). Our results, however, suggest that there may be a fundamental difference in how the two sets of MHC molecules function. Exogenous peptides readily associate with class II MHC molecules on the surface of intact APCs. Similar peptide exchange has been suggested to occur during the recycling of class II MHC molecules. In contrast, in the absence of free β_2m, K^b molecules are largely unreceptive to exogenous peptides.

These differences in the function of the class I and class II MHC molecules imply that there may be differences in their peptide binding sites. It is possible that there is little dissociation of peptides from intact, mature K^b class I MHC molecules or that dissociation occurs and the binding site converts to a nonreceptive state. Peptides can dissociate from class II MHC molecules and it appears that the binding site on the latter molecule remains in an active configuration.

Our conclusion that the intact K^b molecule is relatively unreceptive to peptides may appear to be at variance with other reports that have detected the formation of peptide–MHC complexes after incubations with exogenous peptides for ≤4 hr. We can, however, easily detect the rapid association of OVA with K^b molecules provided that a sufficient concentration of peptide is offered. Under these conditions, the concentration of β_2m in the fetal calf serum-containing culture medium appears to be sufficient to allow direct peptide association (Figs. 4*B* and 6 and unpublished data). Furthermore, there is a second pathway of peptide–MHC molecule association that is dependent on metabolic activity and *de novo* protein synthesis. This latter pathway may involve the entry of peptides into the target cell and association with newly synthesized class I MHC molecules (23). Therefore, these two mechanisms may contribute to the sensitization of target cells with exogenous peptides in other previously described systems. Accordingly, previous assumptions concerning the receptivity of mature class I MHC molecules may be incorrect. Our conclusions are consistent with biochemical measurements of peptide binding to intact class I MHC molecules (25). However, we would caution that it is also possible that other peptides and/or class I MHC

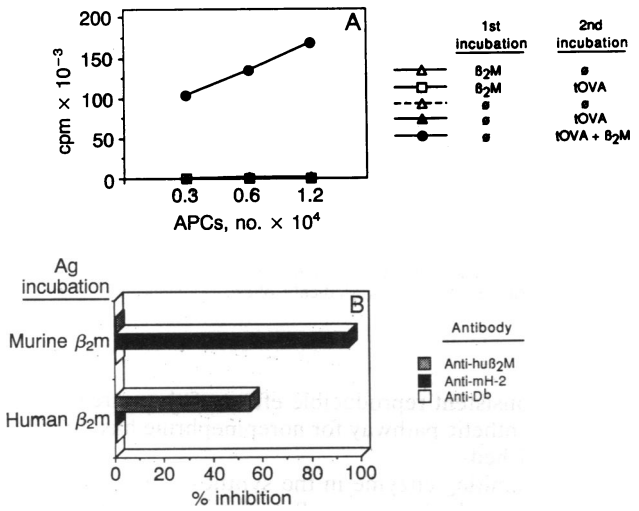

FIG. 6. Exogenous peptide associates with K^b during reassociation of β_2m. (*A*) EL4 APCs were incubated with or without human β_2m (2.5 μg/ml) for 18 hr at 37°C and were washed. APCs were subsequently incubated with or without t-OVA (200 μg/ml) in medium with 1% NMS in the presence or absence of human β_2m (2.5 μg/ml). After 3 hr of incubation at 37°C, the APCs were fixed with paraformaldehyde. Microcultures were prepared as described in the legend to Fig. 1 and contained RF33.70 (anti-OVA-plus-K^b) and EL4 APCs. (*B*) LB27.4 APCs were incubated with 400 μg of t-OVA per ml in Optimem medium supplemented with either 10% NMS or 1% NMS and human β_2m (2.5 μg/ml) for 18 hr at 37°C. Under these latter conditions, we estimate that ≈60–70% of class I MHC molecules are associated with human β_2m (unpublished data). The Ag-exposed APCs were washed, incubated in a saturating amount of 28.14.8S (anti-D^b), M1/42 (anti-murine–murine class I heterodimers), or BBM.1 (anti-human β_2m) for 30 min at 4°C, and were then fixed. Microcultures were prepared with 10^5 NMS cultured APCs or 0.3×10^4 human β_2m cultured APCs and 10^5 RF33.70 cells and were handled as described in the legend to Fig. 1. No additional mAb was added to these cultures. The APCs pulse more strongly in the presence of the human β_2m, and therefore the numbers of APCs added to cultures were adjusted to give approximately equivalent responses; under these conditions the responses of RF33.70 to APCs pulsed in NMS and human β_2m were 35×10^3 and 41×10^3 cpm, respectively, in the control (28.14.8S) groups. The response to APCs not exposed to antigen was 0.4×10^3 cpm.

molecules may behave differently than the OVA peptides and the K^b heterodimer.

Further studies will be necessary to determine whether the results obtained with OVA can be generalized to other Ags. However, it is reasonable to consider that the requirement for free β_2m in the interaction of the K^b molecules and OVA peptides may reflect the general conditions for association of class I MHC molecules with processed peptides. The marked difference in the patterns of association of class I and class II MHC molecules with peptide Ags would then correlate with the distinct functions for which these two molecules have evolved. Class I MHC molecules are expressed on all cells and are meant to display a sample of internally synthesized peptides by the cells for surveillance by cytolytic T cells. It is imperative for the identity of the cells that complexes of class I MHC and internally synthesized peptide be highly stable and/or not easily exchanged with other peptides. In contrast, class II MHC molecules are expressed only on specialized cells concerned with the presentation of foreign protein Ags to initiate the immune response. The capability for dissociation and reassociation of class II MHC molecules and foreign peptides would not be disadvantageous and may result in a more efficient mechanism of presentation of foreign Ags.

Based on our results, we would anticipate that class I MHC molecules will be relatively nonreceptive to exogenous peptides *in vivo*, where the concentration of free β_2m is low. Therefore, our results may have important implications for the use of peptides as vaccines, or as specific competitors, for class I MHC-restricted immune responses *in vivo*.

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