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Reassociation with β_2-microglobulin is necessary for D^b class I major histocompatibility complex binding of an exogenous influenza peptide

(antigen presentation/cytotoxic T lymphocyte/vaccines)

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ABSTRACT **A synthetic peptide corresponding to residues 365–380 of the influenza nucleoprotein (NP365–380) has been previously shown to associate with class I major histocompatibility complex-encoded molecules and to stimulate cytotoxic T lymphocytes [Townsend, A. R. M., Rothbard, J., Gotch, F. M., Bahadur, G., Wraith, D. & McMichael, A. J. (1986) *Cell* 44, 959–968]. We find that intact D^b class I heterodimers on the cell surface are unreceptive to binding this antigen. However, NP365–380 readily associates with D^b molecules on the plasma membrane in the presence of exogenous β_2-microglobulin. In addition, there is a second pathway through which this peptide associates with class I molecules that requires energy and *de novo* protein synthesis. These findings have implications for maintaining the immunological identity of cells and for the use of peptides as vaccines for priming cytolytic T-cell immunity.**

T cells recognize protein determinants in association with major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs) (1). Class I MHC molecules are heterodimeric proteins composed of a transmembrane MHC-encoded polypeptide in association with a non-membrane-spanning polypeptide, β_2-microglobulin. Class II MHC molecules are also heterodimeric proteins but are composed of two MHC-encoded transmembrane polypeptides. Both class I and class II MHC molecules contain binding sites for peptide antigens (Ags) (2–4). Ags in the extracellular fluids are fragmented in the endosomal compartment of APCs and associate with class II MHC molecules (3). Peptides produced from intracellular Ags are generated in a distinct cellular compartment and associate with class I MHC molecules (5–7).

Peptides in the extracellular fluid associate with both class I and class II MHC molecules (3, 8, 9). Under these conditions, peptides bind directly to class II MHC molecules on the APC surface (3, 8, 10). It has been generally assumed that class I MHC molecules also contain free binding sites that may associate with exogenous peptides at the cell surface. By analyzing the presentation of exogenous peptides, it was shown that both classes of MHC molecules are able to present similar, and in some cases identical, antigenic peptides (11–13). Based on these and other data, the peptide binding sites of class I and class II MHC molecules were considered similar and the mechanism by which they bind peptides was considered identical.

In disagreement with this prevalent view, we recently observed that intact K^b class I heterodimers on the cell surface are essentially unreceptive for the binding of ovalbumin (OVA) peptides (14). However, K^b molecules readily bind these peptides in the presence of exogenous β_2-microglobulin (14). There is also a second pathway of peptide association that is dependent on energy and *de novo* protein synthesis (14). These results contrast markedly with the apparent direct association of peptides with class II MHC molecules. The present studies were initiated to determine whether the findings with OVA and K^b molecules could be generalized to other peptides and class I MHC molecules.

MATERIALS AND METHODS

Animals. All mice were purchased from The Jackson Laboratories or were bred by the Animal Resource Division at the Dana–Farber Cancer Center.

Ags. Influenza virus (APR8) was kindly provided by Jonathan Yewdell (National Institutes of Health). A synthetic peptide corresponding to amino acid residues 365–380 of the influenza nucleoprotein (1968 strain) (NP365–380) was synthesized by the Molecular Biology Core Facility at the Dana–Farber Cancer Institute. Human β_2-microglobulin was purchased from Sigma and Calbiochem (American Hoechst, San Diego, CA). Cyanogen bromide-cleaved OVA was prepared as described (14).

Generation of Cytotoxic T Lymphocytes (CTLs). Mice were injected (i.p.) with allantoic fluid (100 μl) from influenza virus-infected eggs. *In vitro* secondary anti-influenza CTLs were prepared essentially as described (15). Briefly, after ≥3 weeks of immunization, influenza immune splenocytes (30×10^6) were cocultured with influenza-infected splenocytes ($15–20 \times 10^6$) in 15 ml of medium in upright 25-cm^2 flasks and were incubated for 5–6 days at 37°C.

Hybridomas and Cell Lines. The RF36.84 hybridoma was generated in a fusion between C57BL/6 anti-influenza-specific CTLs and BW.CD8.7 as described (16). The RF33.70 hybridoma (anti-OVA + K^b specific) has been described (16). R1.1 (C58/J, H-2^k thymic lymphoma) and D1R cells (D^b-transfected R1.1 cells) were kindly provided by Gerald Waneck (Massachusetts General Hospital, Boston). EL4 cells are a C57BL/6 (H-2^b) T-cell tumor.

Hybridoma Cultures. APCs were exposed to Ag as described (14). Hybridoma cultures and interleukin 2 (IL-2) assays were performed as described (14).

RESULTS

Production of an Influenza NP365–380-Specific T-Cell Hybridoma. To determine the generality of our findings with OVA peptides and K^b molecules, we wished to examine the

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Abbreviations: Ag, antigen; APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; FCS, fetal calf serum; MHC, major histocompatibility complex; NP, nucleoprotein; OVA, ovalbumin; IL-2, interleukin 2.

association of a distinct peptide with another class I MHC molecule. For this purpose, we chose to examine the presentation of the influenza peptide, NP365–380, which is an immunodominant epitope recognized by T cells of H-2^b mice. The NP365–380 peptide was one of the first examples of a class I MHC-restricted peptide capable of sensitizing target cells and has been extensively studied (9). This peptide is presented in association with the D^b molecule, whereas the OVA peptides we studied are K^b restricted (6, 9, 16).

Our previous analyses were facilitated by isolation of an OVA-specific, K^b-restricted hybridoma (16). When these cells recognize Ag–MHC complexes, they produce the lymphokine IL-2, which provides a sensitive and quantitative assay to measure Ag presentation (16). Furthermore, since these cells do not require intact and metabolically active APCs, it is possible to use metabolic inhibitors and chemical fixatives to more precisely control the conditions under which Ag exposure and presentation occur (7, 14). We therefore attempted to isolate a similar NP365–380-specific T-cell–T-cell hybrid. For this purpose, splenocytes from influenza immune C57BL/6 mice were stimulated with virus-infected syngeneic cells *in vitro*. The T lymphocytes from these cultures contain strong CTL activity (unpublished data) and were fused to the CD8-transfected BW5147 clone BW.CD8.7. The resulting T-cell hybridomas were screened for their ability to produce IL-2 when stimulated with NP365–380 peptide in the presence of the syngeneic APC EL4. Using this approach we identified the RF36.84 hybridoma.

As illustrated in Table 1, the RF36.84 T-cell hybridoma produces IL-2 when stimulated with NP365–380 and EL4 cells in fetal calf serum (FCS)-containing medium. No response is detected when this cell is stimulated with Ag or the APC alone. To determine the MHC specificity of this hybrid, we used APCs from MHC recombinant inbred mice. RF36.84 is stimulated by NP365–380 in the presence of APCs of B10.A4R but not B10.A3R origin. The reciprocal pattern of presentation of Ag is seen when these APCs are tested with the OVA-specific, K^b-restricted T-cell–T-cell hybrid RF33.70. This latter control demonstrates that the B10.A3R APCs are active in this experiment. These results demonstrate that the recognition of peptide by RF36.84 is MHC restricted and maps the relevant MHC molecule to the telomeric region of the MHC. To precisely identify the relevant class I molecule, we tested the ability of R1.1 cells (H-2^k) that were transfected with the D^b gene to present peptide to RF36.84. As shown in Table 1, RF36.84 is stimulated by peptide and the D^b-expressing APC but not the control APC. We conclude that RF36.84 recognizes NP365–380 in association with D^b molecules.

Table 1. Specificity of an anti-influenza T-cell hybridoma

Exp.	Hybrid	Ag	APC	MHC			cpm × 10^{-3}
1	RF36.84	−	C57BL/6	b	b	b	0.4
		+	C57BL/6	b	b	b	150.6
		−	B10.A3R	b	b/k	d	0.4
		+	B10.A3R	b	b/k	d	0.5
		−	B10.A3R	k	k/b	b	0.7
		+	B10.A4R	k	k/b	b	122.6
1	RF33.70	−	C57BL/6	b	b	b	0.4
		+	C57BL/6	b	b	b	49.3
		−	B10.A3R	b	b/k	d	0.3
		+	B10.A3R	b	b/k	d	27.0
		−	B10.A4R	k	k/b	b	0.6
		+	B10.A4R	k	k/b	b	0.7
2	RF36.84	−	EL4	b	−	b	1.5
		+	EL4	b	−	b	142.7
		−	R1.1	k	−	k	1.2
		+	R1.1	k	−	k	1.4
		−	D^b-R1.1	k	−	k/b	0.6
		+	D^b-R1.1	k	−	k/b	94.5

Duplicate microcultures (200 μl) were prepared with the indicated T-cell hybridoma (10^5) in the presence of the indicated APC and with or without Ag in FCS-containing medium. After 18 hr of incubation at 37°C, an aliquot of culture supernatant (100 μl) was removed and assayed for IL-2 by using HT-2 cells. Data represent the arithmetic mean of cpm of [^{3}H]thymidine incorporated into HT-2 cells. RF36.84 is an anti-influenza + D^b-specific T-cell–T-cell hybrid. RF33.70 is an anti-OVA + K^b-specific T-cell–T-cell hybrid. Where indicated, NP365–380 (5 μM) or CNBr-cleaved OVA (10 μg/ml) was added to cultures with RF36.84 and RF33.70, respectively. Splenocytes (5 × 10^5; Exp. 1) or the indicated cell lines (5 × 10^4; Exp. 2) were added as a source of APCs. The allelic forms of the K, I, and D MHC molecules that are expressed on the APCs are indicated. D^b-R1.1 (D1R) cells are R1.1 cells transfected with the D^b gene.

Presentation of NP365–380 Requires the Presence of Free β_2-Microglobulin. The above experiments were carried out in FCS-containing medium that contained free bovine β_2-microglobulin. To examine the effect of free β_2-microglobulin on the presentation of NP365–380, EL4 APCs were incubated with the peptide in serum-free medium in the presence or absence of exogenous β_2-microglobulin. These APCs were subsequently fixed to prevent further metabolism of Ag and were then tested for their ability to stimulate the RF36.84 hybrid. As shown in Fig. 1, EL4 cells that are incubated with NP365–380 (15 μM) under serum-free conditions do not stimulate RF36.84. However, if purified human β_2-microglobulin is present during the exposure to peptide, the APCs strongly stimulate the T-cell–T-cell hybrid. RF36.84 is not stimulated by APCs exposed to β_2-microglobulin alone (unpublished data). Therefore, under these conditions, the association of peptide with the APC is dependent on the presence of free β_2-microglobulin.

Fetal serum contains high levels of free β_2-microglobulin (17). We therefore examined the association of NP365–380 with EL4 cells in the presence of FCS. As shown in Fig. 1, RF36.84 cells are stimulated by APCs incubated in the presence of NP peptide and 10% FCS. Purified bovine β_2-microglobulin can replace FCS to enable peptide-exposed EL4 cells to stimulate RF36.84 cells (unpublished data).

Presentation of Peptide in the Absence of Added β_2-Microglobulin Requires Energy and Protein Synthesis. In contrast to the above results, APCs that were incubated in very high concentrations of NP365–380 peptide (50–75 μM) in the absence of exogenous β_2-microglobulin stimulated RF36.84 cells (Fig. 2). Therefore, there appears to be a pathway of peptide association that is independent of exogenous β_2-microglobulin. To determine whether this represented the direct association of peptide with class I molecules

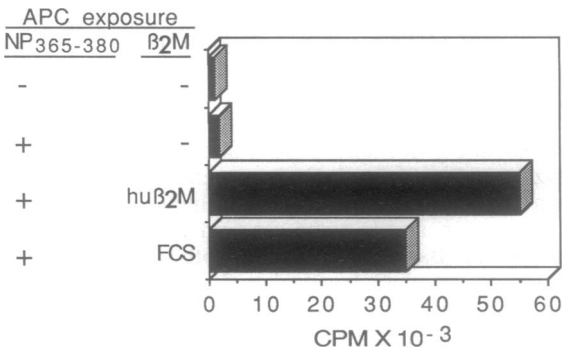

FIG. 1. Association of the NP365–380 peptide with D^b molecules in the presence or absence of exogenous β_2-microglobulin (β_2M). EL4 APCs were exposed to NP365–380 peptide (15 μM) in the presence or absence of purified human β_2-microglobulin (huβ_2M; 25 μg/ml) or FCS (10%) for 3 hr at 37°C and were then washed and fixed with paraformaldehyde. Microcultures were prepared with RF36.84 T-cell–T-cell hybrids and the Ag-exposed and fixed APCs (5 × 10^{-4}) and were handled as described in the legend to Table 1.

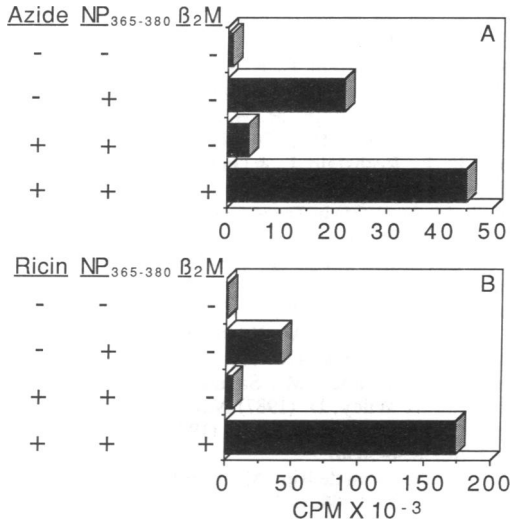

FIG. 2. Effect of metabolic inhibitors on peptide association with D^b molecules. EL4 APCs were exposed to NP365–380 with or without β_2-microglobulin (β_2M) as described in the legend to Fig. 1 except that peptide was added at 50 μM (*A*) or 75 μM (*B*) and, where indicated, azide (30 μM) or ricin (2 nM) was added 30 min prior to, and was present throughout, Ag exposure. Microcultures were prepared and handled as described in the legend to Fig. 1.

on the APC surface, we investigated whether this association was sensitive to metabolic inhibitors, EL4 cells were incubated with azide or ricin and then exposed to high concentrations of NP365–380 in the absence of exogenous β_2-microglobulin. As shown in Fig. 2, the presentation of peptide under these conditions is markedly inhibited by these drugs. Therefore the presentation of NP365–380 under these conditions requires both energy and *de novo* protein synthesis. In contrast, EL4 cells that are treated with these drugs and exposed to peptide in the presence of free β_2-microglobulin strongly stimulate RF36.84 cells.

DISCUSSION

In this report we have examined how the NP365–380 peptide associates with D^b molecules for subsequent presentation to T lymphocytes.

We could not detect the direct association of exogenous NP365–380 peptide with intact D^b heterodimers on the cell surface. We have previously reported an identical observation for the presentation of the OVA258–276 peptide with K^b molecules (14). Thus the same results are observed for two unrelated peptides and two distinct class I MHC molecules. These findings suggest that intact class I MHC heterodimers are generally unreceptive to binding peptides and that free peptide binding sites on class I molecules do not exist to a significant extent on the surface of cells.

Our findings imply that either the class I peptide-binding sites are stably occupied by peptide or, in the absence of a bound peptide, this site exists in an unreceptive state. It is possible that in the absence of peptide, the heterodimer is unstable and the heavy and light chains dissociate. This would be consistent with recent observations on the stability of class I heterodimers in the RMA-S mutant cell line (18). It is also possible that the dissociation of the heterodimer is the rate-limiting event determining the half-life of bound peptides. In either of these two cases, the class I binding site would be lost upon dissociation of the heterodimer. In this context, it is of interest that we have recently detected on the surface of cells a large pool of free class I heavy chain that arises from the dissociation of β_2-microglobulin from assembled class I heterodimers (unpublished observations). It is also conceivable that the conformation of the binding site on intact heterodimers is altered upon peptide dissociation.

In contrast to the above findings, we readily detect the association of the NP365–380 peptide with D^b molecules in the presence of free β_2-microglobulin. This association does not require energy or *de novo* protein synthesis. Again, we have observed the same results in studies of the association of the OVA258–276 peptide with K^b molecules (14). Together, these findings indicate that, as a general rule, peptides may be able to associate with class I MHC molecules on the cell surface in the presence of free β_2-microglobulin.

Our findings imply that the class I peptide binding site is influenced by the reassociation of β_2-microglobulin. This effect must be indirect because β_2-microglobulin is not present in this binding site (19). However, β_2-microglobulin makes numerous physical contacts with residues from the α_1 and α_2 domains of the heavy chain, which form the peptide binding site (19). Accordingly, the reassociation of this light chain may influence the structure and thereby the activity of the binding site. It is possible that under these conditions peptides are binding to class I heterodimers undergoing exchange of their light chain (20, 21) or to previously dissociated class I heavy chains (see above). The latter possibility might be similar to the mechanism through which peptides bind to newly synthesized class I molecules (22).

When cells are exposed to high concentrations of exogenous NP365–380, we can detect the association of this peptide with D^b molecules in the apparent absence of free β_2-microglobulin. In contrast with the above results, this association requires energy and *de novo* protein synthesis. Precisely the same results were observed with the OVA258–276 peptide with K^b molecules (14). A similar effect has been noted for the association of the influenza hemagglutinin peptide, HA508–530, with H-2^d class I molecules (23). This may reflect the association of peptides with newly synthesized class I molecules, a possibility originally suggested by Townsend *et al.* (24). We cannot, however, exclude the possibility that metabolically active cells produce free β_2-microglobulin in sufficient amounts to allow peptides to associate as described above.

The concentration of free β_2-microglobulin in normal adult body fluids is low (17). Under these conditions, our findings predict that exogenous peptides would not associate with class I MHC molecules *in vivo*, except when cells are exposed to very high concentrations of peptides. Accordingly, under physiological conditions the peptides that are displayed in association with class I molecules will be essentially limited to those that are bound during the biosynthetic assembly of MHC molecules. The binding properties of the class I MHC molecule appear thereby to be uniquely suited to preserve the immunologic identity of cells and focus cytolytic immunity to cells synthesizing foreign antigens. In contrast, it is important for class II MHC molecules to be able to bind environmentally derived peptides. The two transmembrane chains of class II molecules may serve to stabilize its antigen binding site until this molecule encounters peptide in endosomes as well as after the dissociation of bound peptide. Indeed, the need to have markedly different capacities to bind environmentally derived peptides may well represent the driving force in evolution that has selected two distinct primary structures for MHC-encoded molecules.

When it was recognized that peptides would associate with class I MHC molecules on cells it was hoped that this could be exploited to develop peptide-based vaccines to stimulate CTL anti-viral immunity. This approach has been successful *in vitro* (15). However, similar attempts to prime CTLs *in vivo* have been generally unsuccessful (25). Our findings provide insight into the probable molecular basis for these results and have clear implications for the development of peptide-based vaccines.

Note Added in Proof. β_2-Microglobulin-dependent association of peptides with K^d, D^b, and D^d molecules has also recently been observed (26, 27).

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