Differential Ability of Isolated H-2 K^b Subsets to Serve as TCR Ligands for Allo-specific CTL Clones: Potential Role for N-linked Glycosylation

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Summary

It is not known whether all forms of cell surface peptide-class I complexes, when bound with relevant peptide antigen, are recognized by T cells. We demonstrate herein that two distinct subsets of the murine H-2 K^b molecule can be separately isolated from H-2^b-expressing cell lines using Y3 mAb immunoaffinity chromatography. Although both isolated K^b subsets were found to be strongly reactive with Y3 mAb by ELISA, one K^b subset is S19.8 mAb reactive (Ly-m11$^+$ K^b subset) and exhibits low reactivity with the M1/42 antibody, while the other subset is negative for the Ly-m11 epitope and highly reactive with the M1/42 antibody (M1/42^{high} K^b subset). More importantly, whereas the M1/42^{high} K^b subset is a very effective ligand for both TCR and CD8, the Ly-m11$^+$ K^b subset could only function as a CD8 ligand, as determined in allo-specific CD8$^+$ CTL clone adhesion and degranulation assays. Peptides acid-eluted from both K^b subsets sensitized K^b-transfected T2 cells expressing "peptide empty" K^b for lysis to a similar extent by allo-CTL clones, indicating that relevant endogenous peptide antigens are not limiting in the Ly-m11$^+$ K^b subset. The major distinction identified between the two K^b subsets is that they differ substantially in their degree of N-linked glycosylation, with the Ly-m11$^+$ subset containing K^b molecules with larger and more complex carbohydrate modifications than the M1/42^{high} subset. The differences in glycosylation may explain the functional differences observed between the two K^b subsets. It is therefore possible that some forms of glycosylation on class I molecules interfere with TCR recognition and may limit CD8$^+$ T cell responses, perhaps under circumstances where peptide antigen is limiting.

Class I MHC-restricted CD8$^+$ CTL recognize a complex of MHC class I heavy chain, β_2-microglobulin (β_2m)[1] and a peptide antigen. An allo-specific CD8$^+$ CTL clone usually recognizes complexes of allo-class I and one or only a small number of specific endogenously processed peptide(s) (1, 2). Collectively, a population of allo-specific CTL respond to allo-class I and a diverse array of endogenous bound peptides (1, 2). Activation of CD8$^+$ CTL requires MHC complex interaction with the TCR, and usually its coreceptor CD8 that provides cosignaling functions (3–5).

We have shown previously that immobilized purified class I MHC alloantigens serve to trigger several allo-specific CD8$^+$ CTL clones and therefore class I alloantigen is a necessary and sufficient ligand for CTL adhesion and activation of a degranulation response (3). When class I is the only ligand on the surface, the CTL adhesion and response depends on the participation of both TCR and CD8 (4). Al-

though specific bound endogenous peptides are required for allo-CTL recognition, it is not known whether all potential forms of a given allo-class I molecule are capable of serving as alloantigens even when bound with relevant peptide(s).

It has been demonstrated that the same class I allele product can be expressed at the cell surface with heterogeneous N-linked carbohydrates (6–8). Although glycosylation on certain murine class I molecules is not required for allo-CTL recognition (9, 10), it is unclear what influence the complexity of N-linked glycosylation on class I may have on CTL recognition, although cell surface sialylation, possibly on class I, influences class I antigen presentation (11).

In the present study, we isolated and characterized two cell surface subsets of murine H-2 K^b from detergent lysates of EL4 and other H-2 K^b-bearing cells. These K^b subsets were found to differ serologically; although both subsets share similar Y3 reactivity, one subset is Ly-m11$^+$ and the other is predominantly reactive with the M1/42 antibody. The two K^b subsets differed dramatically in their abilities to serve as TCR ligands for allo-specific CTL adhesion, and transmembrane signaling as measured by alloantigen-specific degranulation. The two K^b subsets also varied in their N-linked

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[1] Abbreviations used in this paper: β_2m, β_2-microglobulin; DOC, deoxycholate; Endo H, Endoglycosidase H; NANAse, neuraminidase; PL, poly-N-acetyllactosamine; SE, serine esterase.

glycosylation, and how this relates to the functional differences observed between the two K^b subsets is discussed.

Materials and Methods

mAbs. M1/42, a rat anti-mouse mAb (IgG_{2a}) reactive with most H-2 class I antigens (12) was obtained from American Type Culture Collection (ATCC; Rockville, MD). The K^b-specific mAbs used in this study were: Y3 (IgG_{2b}) (13); B8-24-3 (IgG_1) (14); AF6 (IgG_{2a}) (15) obtained from ATCC; and 5F1 (IgG_{2b}), a gift from Dr. L. Sherman (Research Institute Scripps Clinic, La Jolla, CA) (16); and 100-30 (IgG_{2b}), a gift from Dr. U. Hammerling (Memorial Sloan-Kettering Cancer Center, New York) (17). The S19.8 (IgG_{2b}), an anti-mouse β_2m (Ly-m11 epitope) mAb which specifically recognizes β_2m of b strain mice (18), was also a gift from Dr. U. Hammerling. A hamster mAb that recognizes CD3ϵ, 145-2C11 (19), was obtained from ATCC. The anti-CD8 mAb, 3.168 (IgM) (20), was a gift from Dr. A. O'Rourke (Research Institute Scripps Clinic, La Jolla, CA).

Enzymes. Recombinant *Streptomyces plicatus* Endoglycosidase H (Endo H) was purchased from Boehringer Mannheim Biochemica and Endoglycosidase F (Endo F) purified from *Flavobacterium meningosepticum* was purchased from Du Pont Company (Wilmington, DE). Type V neuraminidase (NANAse) from *Clostridium perfringens* was purchased from Sigma Immunochemicals (St. Louis, MO).

Tumor Cell Lines. The murine T cell lymphoma cell line EL4 (H-2^b) was obtained from ATCC. ANA-1, a macrophage cell line of C57BL/6 origin (21), was a gift from Dr. M. Belosevic (University of Alberta, Edmonton, Canada). The murine pre-B cell line, R8 (H-2^{bxd}) (22) was provided by Dr. R. Zeff (University of Connecticut, Farmington, CT). The human transfected cell line T2K^b (23) was a gift from Dr. P Cresswell (Yale University, New Haven, CT).

Cloned CTL. The K^b allo-specific CTL clones 11, 35, 6, 13 and AB.1 have been well documented (3, 24). The D^d allo-specific CTL clone 10/1 was derived from C57BL/6 spleen cells primed in vitro with irradiated (3,000 rad) Balb/c spleen cells and cloned 1 wk later by limiting dilution in the presence of 5% Con A supernatant. All the allo CTL clones were maintained as previously described (3, 24).

Isolation and ELISA of H-2 K^b Subsets. The H-2 K^b was purified from various cell lines by immunoaffinity chromatography as described (25) with modifications. EL4 cells were propagated in vivo as ascites, while the ANA-1 and R8 cells were grown in 5% FCS-DMEM. Detergent lysates from 1.0–1.5 × 10^{10} cells were passed over a Y3 affinity column preceded by a Sepharose 4B precolumn. Two sequential elutions were performed immediately after the column washes (25). The first elution buffer, 0.5% DOC, 0.65 M NaCl, 10 mM Tris, pH 8.3–8.5, has been described previously (25). Following elution with this buffer, a second elution is done using a buffer of higher pH: 0.5% DOC, 0.15 M NaCl, 15 mM Na$_2$CO$_3$, pH 10.5. For some preparations, fractions of the first (pH 8.5) or second (pH 10.5) elution showing strong ELISA reactivity with the K^b-specific mAb Y3 as first antibody were separately pooled and stored at −20°C. Direct ELISA of affinity-purified H-2 K^b was performed as previously described (25). Immunoaffinity purifications of K^k and I-E^d, I-A^d molecules used in this study were as described previously (25). For monitoring the purification of cell surface K^b molecules, 20 × 10^6 EL4 cells were ^{125}I-labeled by lactoperoxidase/oxidase-catalyzed iodination (26), and their lysates added to the routine EL4 preparation as tracer.

Assay of CTL Binding and Degranulation. CTL adhesion and degranulation responses to purified class I and coimmobilized proteins has been previously described in detail (4). 1–2 × 10^5 ^{51}Cr-labeled CTL were incubated for 4 h at 37°C on protein-bearing plate wells and unbound cells were then removed. In experiments employing fluid phase anti-CD3 mAb, the antibody was added to the cells in suspension and the cells immediately placed into the well. The anti-CD8 mAb blocking experiment was performed by incubating CTL with the antibody at the indicated concentration at room temperature for 30 min. Cell binding was calculated as: percent cell bound = 100 × [(cpm bound)/(total cpm-spontaneous cpm)]. Experiments were done in triplicate for each condition unless specified.

Degranulation by the CTL was assessed by measuring the serine esterase (SE) activity released into the medium by the N'-benzyloxycarbonyl-L-lysine thiobenzyl ester assay as described (3, 4). Determination of CTL binding and SE release was done simultaneously from the same wells. The SE release assay was allowed to proceed for 20 min and OD$_{405nm}$ was determined. SE release is expressed as ΔOD$_{405nm}$ = OD$_{405nm}$(CTL + immobilized class I) − OD$_{405nm}$(CTL + wells blocked with 2% FCS in PBS). Determinations were done in triplicate for each condition unless specified.

Acid Extraction of Peptides from Purified K^b Subsets. Peptides associated with purified K^b were extracted by TFA (27). Peak fractions of pH 8.5-eluted (Ly-m11$^+$) and pH 10.5-eluted (M1/42^{high}) subsets were separately pooled. The pools were serially diluted, ELISA using Y3 as first antibody was performed and equivalent amounts of Y3-reactive K^b (defined as equal value of ELISA OD value × sample volume) were subjected to acid extraction with a final concentration of 0.05% TFA. The samples were centrifuged in Centricon-10 microconcentrators, filtrates (<10^4 mol wt) freeze dried, resuspended in 0.1% TFA-H$_2$O, and separated on C$_{18}$ reverse phase HPLC. Both pH 8.5 and 10.5 elution buffers were subjected to identical treatment in parallel as mock-treated controls.

HPLC Separation of Extracted Peptides. Peptide were separated on a narrow-bore C$_{18}$ (2 mm × 25 cm) column set up on a Beckman System Gold HPLC. The solvents were A: 0.1% TFA-H$_2$O; B: 0.09% TFA, 80% acetonitrile, 20% H$_2$O. A linear gradient of acetonitrile was applied with the gradient rate of 0.8% acetonitrile/min and a flow rate of 0.2 ml/min. Fractions corresponding to the 20–50% acetonitrile gradient were pooled, lyophilized, washed, and reconstituted in 50 μl D-PBS for the sensitization of T2K^b cells for allo-CTL lysis.

Cytotoxicity Assay. Peptides extracted from purified K^b were tested for their ability to sensitize T2K^b cells for lysis by allo-specific CTL clones. ^{51}Cr-labeled T2K^b cells were resuspended in assay medium at a density of 3 × 10^6/ml. A volume of 50 μl of the cell suspension was incubated with various dilutions of peptides isolated from purified K^b subsets at 37°C for 45 min. Then the T2K^b cells were diluted to 1 × 10^5/ml with assay medium and 100 μl was incubated with various CTL clones at a 5:1 E/T ratio for 5 h in V-bottom plates in triplicate. Percent specific ^{51}Cr release was calculated as: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Results are expressed as mean % specific ^{51}Cr release ± SD.

SDS-PAGE. The purified H-2 K^b subsets were analyzed by 10% or 12.5% SDS-PAGE under reducing conditions (28). Gels were stained by Coomassie blue. Autoradiography was also carried out to visualize the ^{125}I-labeled proteins in the gel.

Endo H, Endo F, and NANAse Treatment of Purified H-2 K^b Subsets. The isolated H-2 K^b proteins were acetone precipitated in the presence of BSA as carrier, and the precipitates were dissolved in 50 μl of their respective enzyme incubation buffers (29). Samples were incubated with 3–6 mU of Endo H or 0.5–1.0 U of Endo F as previously described (10, 29). NANAse treatment

of samples was performed with 0.5–0.6 U of NANAse at 37°C for 4 h (11).

Results

Isolation of Two Serologically Distinct K^b Subsets from H-2^b–expressing Cell Lines. Standard protocols for immunoaffinity purification of murine class I MHC molecules from cell detergent lysates typically involve elution of the antigens from the affinity column at pH 8.5 in 0.5% DOC detergent (25, 30). However, we have found that not all K^b is eluted from a Y3 mAb column using standard elution conditions at pH 8.5 (data not shown). If an elution buffer of pH 10.5 is used immediately following the pH 8.5 elution buffer, a second immunoreactive K^b peak can be isolated from several H-2^b–expressing cell lines as determined by solid phase ELISA using Y3 as first antibody (Fig. 1, data not shown). We also analyzed individual eluted fractions by ELISA with the S19.8 mAb which recognizes the Ly-m11 epitope on β_2m when associated with K^b (22). S19.8-reactive material eluted coincidently with the first Y3-reactive peak (peak 1), but not the second (peak 2), indicating that two serologically distinct K^b subsets can be separately isolated from H-2^b–expressing cells using a Y3 affinity column (Fig. 1). The two K^b subsets can be isolated from various cell lineages including T cell lines e.g., EL4 (Fig. 1 *a*), macrophage/monocyte lines, e.g., ANA-1 (Fig. 1 *b*) and also from a pre-B cell line, R8 (Fig. 1 *c*). To further characterize the two K^b subsets, both serologically and functionally, we have gone on to use K^b subsets isolated from EL4 because of the greater yield of both subsets from this cell line.

ELISA Reactivity of Isolated H-2 K^b Subsets. The strongly Y3-reactive fractions of peak 1 and peak 2 isolated from EL4 cells were pooled separately and ELISA reactivity with several K^b-reactive antibodies was determined. From the ELISA results, the mAbs can be grouped into three categories (Table 1). The first group includes Y3, 100-30, 5F1, and AF6 which react strongly to both K^b subsets. Y3 is reported to recognize K^b irrespective of association with β_2m or specific peptides (31, 32). The Y3 reactivity by ELISA is high for the pH 8.5- and 10.5-eluted K^b (Table 1). In contrast to Y3, the reactivity of 5F1 with K^b is dependent on associated peptide (31). Reactivity of this mAb is found with both K^b subsets at comparable levels, suggesting that both K^b subsets have bound peptides. Furthermore, both subsets also react with 100-30 and AF6 which recognize peptide-dependent conformational epitopes of K^b (33).

A second mAb group only includes S19.8. This antibody recognizes K^b associated with the β_2m expressed by C57BL/6 and related mouse strains and a bound peptide (18, 22) and reacts exclusively with peak 1, but not the peak 2 subset of K^b and therefore readily discriminates between these two subsets.

A third group includes M1/42 and B8-24-3 which are strongly reactive with peak 2, but react weakly with peak 1. M1/42 mAb is also strongly reactive with peak 2 of both ANA-1 and R8 cells but weakly reactive with peak 1 (data not shown). Since M1/42 mAb reactivity requires mouse
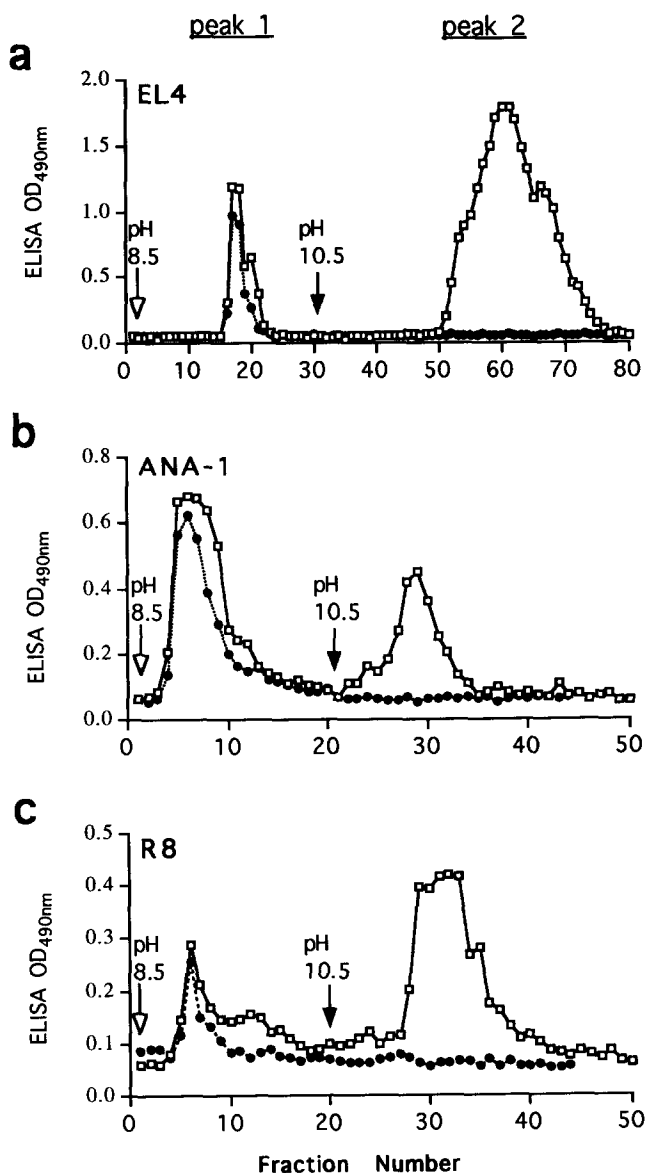

Figure 1. Isolation of two serologically distinct K^b subsets from H-2K^b expression cells. K^b molecules were isolated from (*a*) EL4, (*b*) ANA-1, and (*c*) R8 cells by Y3 mAb immunoaffinity chromatography. Elutions of K^b from Y3 mAb column were performed by sequential application of pH 8.5 and 10.5 buffers. Open and solid arrows indicate the start of pH 8.5 and 10.5 elutions, respectively. Each fraction (1:20 dilution) was assayed for Y3 (□) and S19.8 (Ly-m11) (●) mAb reactivity by solid-phase ELISA. Purified mAb Y3 (2.1 mg/ml) was diluted 1:500, and S19.8 culture supernatant was at a 1:4 dilution. Results are expressed as an average of OD$_{490\text{nm}}$ of duplicate wells.

β_2m to be associated with murine class I molecules (34), these results demonstrate that peak 2 contains class I molecules with associated murine β_2m. Therefore both isolated subsets contain K^b associated with murine β_2m. The B8-24-3 mAb recognizes a conformational epitope dependent on β_2m and peptide association, providing further evidence that peak 2 contains peptide–MHC complexes. Based on the serological characteristics of the two K^b subsets determined by

Table 1. *ELISA Reactivity of H-2 K^b Subsets Isolated from EL4 Cells* *

mAb[‡]	Peak 1 (pH 8.5 elution)	Peak 2 (pH 10.5 elution)
Y3	0.861 ± 0.051[§]	0.885 ± 0.038
5F1	0.361 ± 0.028	0.488 ± 0.024
100-30	0.590 ± 0.050	0.842 ± 0.071
AF6	0.662 ± 0.056	0.722 ± 0.091
S19.8 (Ly-m11)	0.852 ± 0.054	0.005 ± 0.012
M1/42	0.033 ± 0.016	0.917 ± 0.026
B8-24-3	0.153 ± 0.023	0.812 ± 0.083

* ELISA reactivity of isolated EL4 K^b subsets to various mAbs was determined. From a representative preparation, ELISA peak fractions of pH 8.5- and pH 10.5-eluted K^b were pooled separately, diluted 1:20 into D-PBS for all assays.
‡ First antibody used in ELISA assay: Y3 (2.1 mg/ml) and M1/42 (1.5 mg/ml) were purified and diluted 1:500 in 2% FCS-PBS; 5F1 was used as ascites at a 1:100 dilution; 100-30, AF6, B8-24-3 and S19.8 (Ly-m11) were used as culture supernatants in 5% FCS-RPMI at 1:4 dilution.
§ Results were expressed as mean OD_{490nm} ± SD of triplicate wells.

solid-phase ELISA, we operationally define the pH 8.5-eluted K^b subset as Ly-m11 $^+$ and the subsequent pH 10.5-eluted K^b subset as M1/42^{high}.

Allo-specific CTL Clones Bind and Are Triggered by the M1/42^{high} but Not the Ly-m11 $^+$ K^b Subset. Due to the serological differences detected in the previous ELISA studies (Table 1) between the two K^b subsets, we tested the ability of each isolated subset immobilized on solid-phase to stimulate antigen-specific degranulation by K^b allo-specific CTL clones (3). We found that the M1/42^{high} K^b subset stimulated degranulation of allo-specific CTL clones as measured by SE release, whereas the Ly-m11 $^+$ K^b subset did not (Fig. 2 *a*). The degranulation response of the three CTL clones, elicited by the M1/42^{high} K^b subset, is comparable to that triggered by K^b-bearing EL4 allogeneic target cells (3, and data not shown).

When binding of the CTL clones to immobilized Ly-m11 $^+$ and M1/42^{high} K^b subsets was examined, similar results to the SE release assay were obtained (Fig. 2 *b*). Substantial binding, 40–60% of the input CTL, was observed to the immobilized M1/42^{high} K^b subset. In contrast, only minimal binding above background, 1–5%, could be detected with the immobilized Ly-m11 $^+$ K^b subset. Equivalent densities of Y3-reactive K^b of both subsets were used in the binding and SE assays, while the contrasting S19.8 and M1/42 reactivity of each subset was also confirmed by ELISA (Fig. 2 *c*). Additional allo-specific CTL clones 35 and 13 (3, 24), also displayed strong adhesion and response to the M1/42^{high} K^b but no adhesion or response to the Ly-m11 $^+$ K^b subset of immunoaffinity purified K^b on solid-phase (data not shown). Taken together, the results in Fig. 2 suggest that there are functional differences between the Ly-m11 $^+$ and
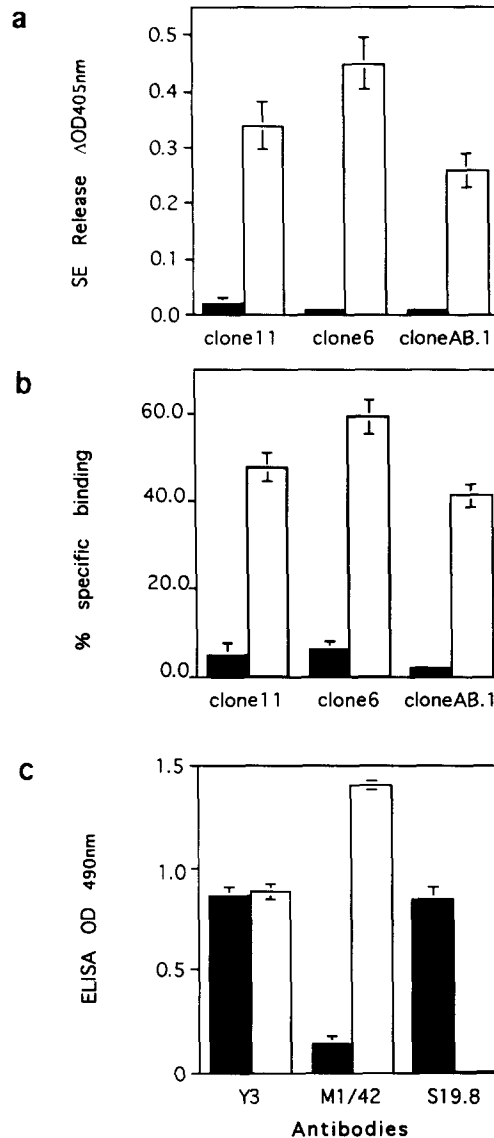
Figure 2. Allo-specific CTL clones bind and are triggered by the M1/42^{high}, but not Ly-m11 $^+$ K^b subset. Antigen-specific SE release (*a*) and binding (*b*) of three K^b allo-specific CTL clones in response to immobilized Ly-m11 $^+$ (*solid bar*) and M1/42^{high} (*open bar*) K^b subsets were determined. 2 × 10^5 of each CTL clone were added to each well and incubated for 4 h. Results are expressed as mean ± SD. The same Ly-m11 $^+$ (*solid bar*) and M1/42^{high} (*open bar*) K^b subsets were assayed for their immunoreactivity with Y3, M1/42 and S19.8 mAbs in parallel by ELISA (*c*) at a density identical to that used in the above CTL binding and SE release experiments. Results are expressed as mean OD_{490nm} ± SD.

M1/42^{high} K^b subsets; the allo-CTL bind and degranulate in response to the M1/42^{high} K^b subset, but not the Ly-m11 $^+$ K^b subset.

Dose-response Relationship of CTL Recognition of K^b Subsets. The Ly-m11 $^+$ and M1/42^{high} K^b subsets were serially diluted and immobilized separately on solid-phase and assayed for binding and stimulation of degranulation of allo-specific CTL clone 11. At the highest density shown, both K^b subsets reached equivalent saturating densities as detected by solid-

phase Y3 ELISA (data not shown). The CTL SE release response to the M1/42^{high} K^b subset shows a titration curve that includes a threshold density for stimulation and rises to a maximal response at saturating density (Fig. 3). Adhesion of the CTL clones to the immobilized M1/42^{high} K^b subset closely paralleled the dose-response curve of SE release. In contrast, the Ly-m11$^+$ K^b subset could not be bound or stimulate the CTL clone at any density, even when the Ly-m11$^+$ K^b subset is at a high and saturating density (Fig. 3). Other K^b allo-specific CTL clones including clone 35 and AB.1 also were bound to and were stimulated by the M1/42^{high} K^b subset, but did not bind or respond to the Ly-m11$^+$ K^b subset (data not shown).

The Ly-m11$^+$ Subset K^b Molecules Do Not Inhibit CTL Recognition of the M1/42^{high} K^b Subset. We tested the possibility that the Ly-m11$^+$ K^b subset contained inhibitory activities that could prevent or interfere with recognition of K^b alloantigen by CTL. As expected, plate wells immobilized with the Ly-m11$^+$ K^b subset did not stimulate the CTL, whereas the M1/42^{high} K^b subset did stimulate significant antigen-specific CTL degranulation (Fig. 4). Coimmobilization of the nonstimulatory Ly-m11$^+$ K^b subset with the M1/42^{high} K^b did not inhibit the antigen-specific CTL degranulation response to the M1/42^{high} K^b subset (Fig. 4). Since the Ly-m11$^+$ K^b subset does not inhibit CTL recognition of M1/42^{high} K^b subset, it is therefore unlikely that inhibitory activities are present within the Ly-m11$^+$ K^b subset that could readily explain the lack of response to the Ly-m11$^+$ K^b subset by allo-specific CTL.

The M1/42^{high} but Not the Ly-m11$^+$ K^b Subset Serves as a CTL TCR Ligand. The failure of the Ly-m11$^+$ K^b subset to trigger allo-CTL could be due to an inability to engage and trigger TCR and/or CD8 molecules on the CTLs (4). We have demonstrated that when a suboptimal density of purified class I alloantigen is not sufficient to trigger a full CTL response, it can still activate via TCR engagement,
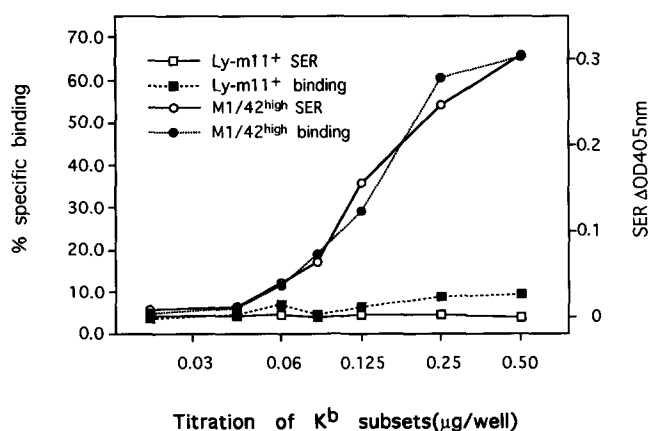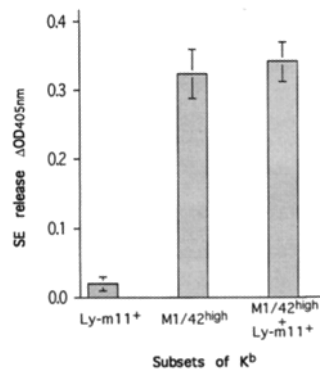
Figure 4. The Ly-m11$^+$ K^b subset does not inhibit recognition of the M1/42^{high} K^b subset by an allo-specific CTL clone. Two K^b subsets containing an equivalent amount of Y3-reactive K^b were diluted 1:20 separately in D-PBS and immobilized individually on the plate. For the mixing experiment, both K^b subsets were diluted together into D-PBS in which each K^b subset is 1:20 diluted, and coated on the plate. Clone 11 SE release triggered by Ly-m11$^+$, M1/42^{high}, or the mixture of both K^b subsets was determined.

"avidity enhanced" CD8 binding to coimmobilized non-Ag class I molecules (4), resulting in enhanced CTL binding and degranulation response (4). If the Ly-m11$^+$ K^b subset can serve as a TCR ligand, it should trigger enhanced CD8-dependent CTL binding and possibly a degranulation response when coimmobilized with non-Ag class I molecules.

When the Ly-m11$^+$ K^b (possible TCR ligand) was coimmobilized with K^k (non-Ag class I, CD8 ligand) we could not detect TCR-triggered CTL binding or degranulation of CTL clone 11 (Fig. 5, *a* and *b*). At no density could the Ly-m11$^+$ K^b subset trigger clone 11 CTL with or without coimmobilization with non-Ag class I (data not shown). In contrast, although the M1/42^{high} K^b subset at a suboptimal density was not sufficient to trigger the allo-CTL clone 11 to bind and degranulate alone, it triggered clone 11 to bind to coimmobilized K^k and degranulate, indicating that the M1/42^{high} K^b subset serves as a TCR ligand (Fig. 5, *a* and *b*). The TCR-triggered binding or degranulation of the CD8$^+$ CTL clone was not observed when either K^b subset was coimmobilized with class II MHC control molecules (Fig. 5, *a* and *b*). These results suggest that the immunoaffinity purified M1/42^{high} K^b subset serves as a TCR ligand, upregulating CD8 adhesion and signaling function, but the Ly-m11$^+$ K^b subset does not serve this function for clone 11 and all of a number of other K^b alloantigen-specific CTL clones tested (Fig. 5 and data not shown).

Both the Ly-m11$^+$ and M1/42^{high} K^b Subsets Are CD8 Ligands. Since the preceding results showed that only the M1/42^{high} K^b subset could serve as a TCR ligand, we sought to determine whether either subset could serve as a CD8 ligand. It has been shown that soluble anti-TCR antibody activates adhesion of CD8$^+$ CTL to class I, but not class II MHC proteins (35). The adhesion is mediated by CD8, since it is effectively inhibited by anti-CD8 antibody (35). When the D^d allo-specific CTL clone 10/1 was treated with soluble anti-CD3 antibody, 145-2C11, it bound and degranulated in response to immobilized non-Ag class I (K^k), but not class II (I-A^d) proteins (Fig. 6, *a* and *b*). A substantial and similar degree of TCR-activated CTL binding was observed for both immobilized Ly-m11$^+$ and M1/42^{high} K^b subsets (Fig. 6 *a*), and CTL binding to both K^b subsets could be effectively

Figure 3. Dose-response relationship of CTL recognition of K^b subsets. Both K^b subsets were serially diluted separately and immobilized on plastic. 10^5 clone 11 CTL were added and incubated for 4 h. Results of both CTL binding and SE release were averages of duplicate wells. The variation was less than 10% between wells.

a

b
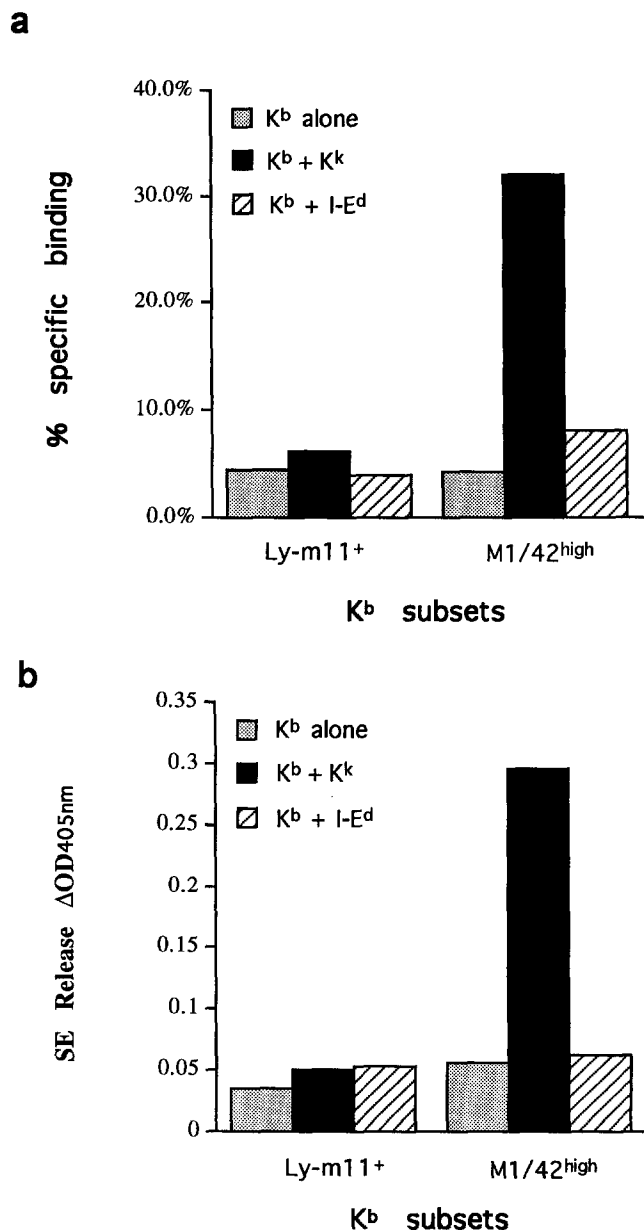

Figure 5. The M1/42^high, but not the Ly-m11+ K^b subset, can serve as a TCR ligand. Both the Ly-m11+ and M1/42^high K^b subsets (0.07 μg/well) were separately immobilized alone, or coimmobilized with H-2 K^k (0.15 μg/well) or I-E^d (0.21 μg/well) as indicated. The wells were blocked with 2% FCS in D-PBS. Clone 11 CTL were added to wells at 1.5 × 10^5 cell/well, incubated for 4 h at 37°C, and binding (a) and SE release (b) determined. Data were expressed as the mean of triplicate wells.

blocked by anti-CD8 mAb (Fig. 6 a, inset). In addition, strong degranulation responses to immobilized K^b subsets were observed, with the Ly-m11+ subset providing somewhat stronger CD8 costimulation than the M1/42^high K^b subset (Fig. 6 b). From these observations, we concluded that both K^b subsets can serve as CD8 ligands, and the Ly-m11+ K^b subset may possibly deliver more efficient signals through CD8 to activate CTL degranulation.
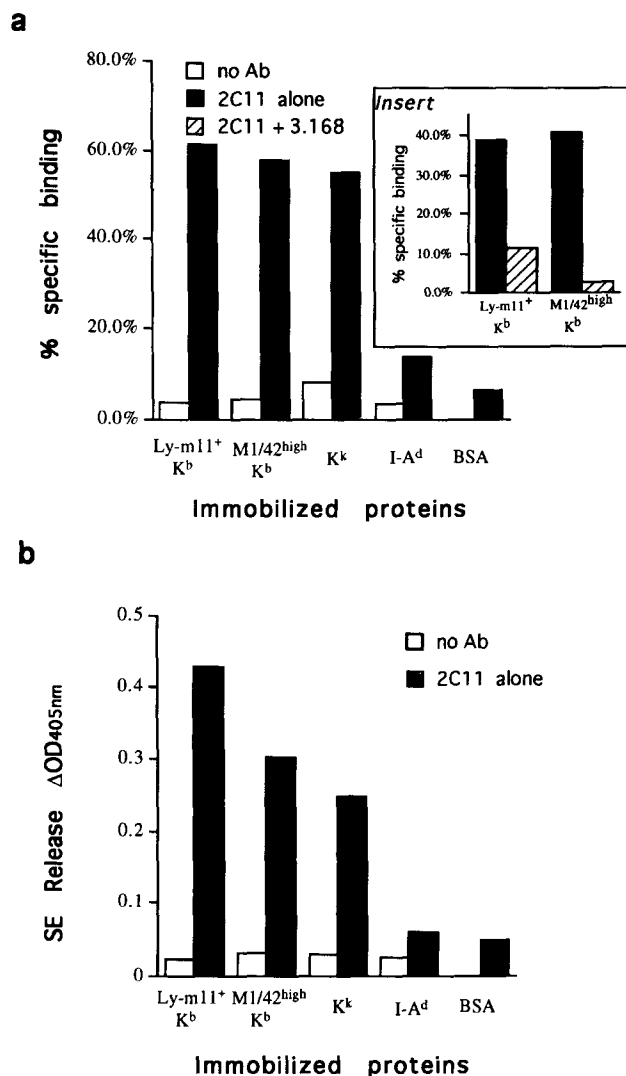
a

b

Figure 6. Both the Ly-m11+ and M1/42^high K^b subsets can serve as CD8 ligands. The Ly-m11+ and M1/42^high K^b subsets (both at a density of 0.15 μg/well), H-2 K^k (0.16 μg/well), I-A^d (0.20 μg/well), or BSA (100 μg/well) were separately immobilized on the wells as indicated, and blocked with 2% FCS in D-PBS. The D^d allo-specific CTL clone, 10/1, was loaded into the wells at 1.0 × 10^5 cell/well. Immediately before loading, clone 10/1 cells were treated with 0.25 μg/ml 2C11 mAb (solid bar), or without antibody treatment (open bar). CTL clone 10/1 binding (a) and SE release (b) were determined. In a separate experiment, the effect of anti-CD8 mAb on clone 10/1 binding was tested (a, inset). Clone 10/1 were incubated at room temperature with 3.168 mAb at 1:100 dilution of ascites for 30 min before the addition of 2C11 mAb. Clone 10/1 binding in the absence of 2C11 was 3.5% (not shown). In the presence of 2C11 mAb, clone 10/1 adhesion with (hatched bar) or without (solid bar) 3.168 mAb treatment was determined. All data were expressed as the mean of triplicate wells. Binding and SE release of clone 10/1 in the absence of mAb 2C11 in the BSA-coated wells were determined as background and subtracted from the experimental groups.

The Lack of TCR Recognition of Ly-m11 + K^b Subset Is Not Due to the Absence of Relevant Bound Endogenous Peptides. That most allo-specific CTL recognize an allo-class I molecule bound with specific endogenously processed peptide(s) (1, 2) suggested the possibility that the functional differences exhibited

by the Ly-m11$^+$ and M1/42^{high} K^b subsets could reflect differences in the composition of endogenous peptides bound by the two K^b subsets. To test this possibility, we acid-eluted endogenous peptides from equivalent amounts of the two isolated K^b subsets, based on their Y3 ELISA reactivity, and assayed them for the ability to sensitize T2K^b cells for lysis by K^b-specific allo-CTL (1, 36). The T2K^b cells are believed to express peptide "empty" K^b molecules and they are efficient at binding and presenting exogenous peptide antigens (23). Lysis of T2K^b cells by allo-specific CTL clone 35 and clone 11 is dependent on endogenous peptides eluted from isolated K^b (Fig. 7, a and b). Peptides eluted from both Ly-m11$^+$ and M1/42^{high} K^b subsets sensitized T2K^b for lysis to comparable levels, by clone 35 (Fig. 7 a) and clone 11 (Fig. 7 b).

Titration of the eluted peptides from the K^b subsets for the T2K^b sensitization indicates that the yields of the relevant peptides mediating allorecognition for clone 11 (Fig. 7 b) and clone 35 (data not shown) are similar for the Ly-m11$^+$ and M1/42^{high} K^b subsets. From these results, we conclude that the relevant peptides for recognition by the allo-specific CTL clones are associated with both the Ly-m11$^+$
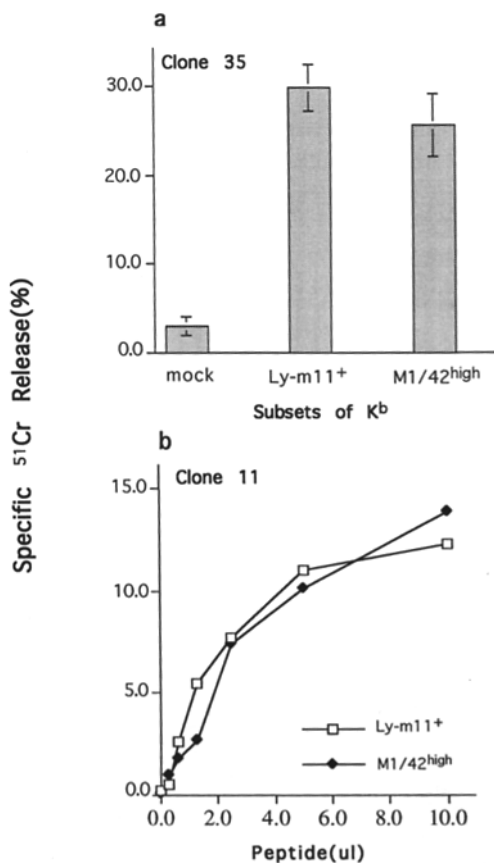
and M1/42^{high} K^b subsets. Furthermore, the appropriate peptides do not appear to be limiting in the Ly-m11$^+$ subset, hence it is unlikely that associated peptides provide an explanation for the differences in functional activities of the Ly-m11$^+$ and M1/42^{high} K^b subsets.

The Ly-m11$^+$ and M1/42^{high} K^b Subsets Differ in Their N-Linked Glycosylation. We attempted to determine the biochemical basis for the observed serological and functional differences between the two K^b subsets. The SDS-PAGE analysis with Coomassie blue staining revealed a 47-kD K^b heavy chain (Fig. 8 a, lane 2, solid arrow) and a 12-kD β_2m (not shown) in the M1/42^{high} K^b subset. Two major bands were seen in the Ly-m11$^+$ K^b subset; one band at 51 kD (Fig. 8 a, lane 1, barbed arrow) with a barely visible smear on top and another band at 43 kD (Fig. 8 a, lane 1, open arrow). The identity of the 43-kD band is unclear, however it is Endo H and Endo F insensitive (data not shown) and is therefore likely to be a contaminant such as actin, which has been seen in class I immunoprecipitations (29, 37). For the Ly-m11$^+$ K^b subset, the β_2m is less readily seen in Coomassie staining, but is visualized by silver staining (data not shown).

When the K^b subsets were purified from EL4 cells labeled with ^{125}I and visualized by autoradiography, a clear difference in molecular mass between the two K^b subsets was again observed. The M1/42^{high} K^b is a 47-kD band (Fig. 8 b, lane 4); in contrast, the Ly-m11$^+$ K^b subset was visualized as a broad 51–56-kD band likely to be made up of multiple closely migrating bands (Fig. 8 b, lane 1). This 51–56-kD broad band most probably includes the 51-kD band on the Coomassie blue staining gel in Fig. 8 a. Since both EL4 K^b subsets were labeled by ^{125}I, we presume that they are both cell surface forms of K^b. Higher molecular mass H-2K^b

Figure 7. The lack of TCR recognition of Ly-m11$^+$ K^b is not due to a lack of relevant bound endogenous peptides. (a) Clone 35 killing of ^{51}Cr-labeled T2K^b cells sensitized with 10 μl of peptides isolated from both K^b subsets with equivalent Y3-reactivity. (b) Different volumes of eluted peptides isolated from Ly-m11$^+$ or M1/42^{high} K^b subsets were used to sensitize T2K^b cells for killing by a K^b allo-specific CTL clone, 11. Percent specific lysis is expressed as the mean of triplicate wells.
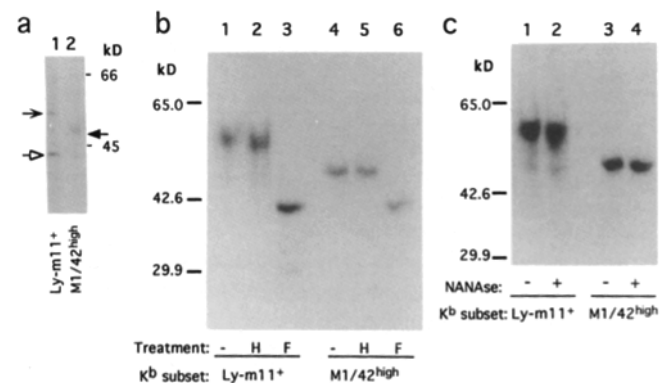

Figure 8. Biochemical analysis of the Ly-m11$^+$ and M1/42^{high} K^b subsets. (a) SDS-PAGE analysis of the purified K^b subsets. Equivalent amounts of the Y3-reactive Ly-m11$^+$ (lane 1) and M1/42^{high} (lane 2) K^b subsets were analyzed on 10% SDS-PAGE with Coomassie blue staining. Molecular mass markers are shown on the right. (b) Endo H and Endo F treatment of the K^b subsets. Both K^b subsets were treated with 6 mU Endo H (H) or 0.8 U Endo F (F) or mock treated ($-$) at 37°C for 20 h, analyzed on 10% SDS-PAGE, and visualized by autoradiography. Molecular mass markers are as shown. (c) NANAse treatment of the K^b subsets. The K^b subsets were treated with 0.5 U NANAse ($+$) or mock treated ($-$) at 37°C for 4 h, analyzed on 12.5% SDS-PAGE and visualized by autoradiography.

molecules with a similar migration and banding pattern to that observed in the EL4 Ly-m11$^+$ K^b subset have been identified in immunoaffinity purified preparations of K^b from the B/6 murine leukemia cell line RBL-5 and may represent similar K^b species (38, 39).

Since the apparent differences in migration of the Ly-m11$^+$ and M1/42^{high} K^b subsets on SDS-PAGE could be due to differences in glycosylation, we performed Endo H, Endo F, and NANAse treatment of both K^b subsets. After Endo F treatment, both the 51–56-kD broad band in the Ly-m11$^+$ K^b subset (Fig. 8 b, lane 1) and the 47-kD band in the M1/42^{high} K^b subset (Fig. 8 b, lane 4) shifted in mobility to a position corresponding to 40 kD (Fig. 8 b, lanes 3 and 6). This result suggests that: (a) both K^b subsets are N-linked glycosylated, and (b) their peptide backbones are of the same or very similar size, making it very unlikely that the difference in molecular mass of the two K^b subsets is due to differential splicing (40). Both subsets were found to be resistant to Endo H treatment (Fig. 8 b, lanes 2 and 5), indicating that the N-linked carbohydrates on both subsets are of a mature or complex form. Upon NANAse treatment, the 51–56-kD band of the Ly-m11$^+$ K^b subset shifted only very slightly towards the gel bottom (Fig. 8 c, lane 2). This suggests either that sialylation is not the major factor responsible for the mobility difference between the Ly-m11$^+$ and M1/42^{high} K^b subsets, or there exist sialic acid-containing carbohydrates on the Ly-m11$^+$ K^b subset that are resistant to NANAse treatment (41). Occasionally, upon longer exposure, we see a faint 47-kD band that corresponds to the size of the M1/42^{high} K^b band in the Ly-m11$^+$ K^b subset (Fig. 8 c) which may or may not be Ly-m11 reactive or possibly represents some contaminating M1/42-reactive K^b. In summary, the Ly-m11$^+$ and M1/42^{high} K^b subsets differ in their N-linked glycosylation in addition to their serological and functional differences. The observed variance in glycosylation between the two K^b subsets may be responsible for their observed functional differences.

Discussion

We have demonstrated herein that two immunoaffinity purified K^b subsets differ in their serology, glycosylation and ability to trigger allo-specific CTL. The serological characteristics of the two K^b subsets (Table 1) indicate that each contains K^b heavy chain associated with β_2m as the epitopes recognized by S19.8, M1/42 and AF6 mAbs are dependent on class I heavy chain association with murine β_2m (22, 31, 34). Furthermore, since both K^b subsets are reactive with several mAbs that are dependent on peptide association with K^b (31–33), both subsets contain endogenous peptides. Complexes of class I MHC molecules with different endogenous peptides can be distinguished from each other by allo-antibodies (27, 31–33). These serologically defined differences may be due, in part, to subtle peptide-dependent conformational variations in the class I heavy chain (31), or to differences in the size of exposed peptide side chains that may affect antibody binding (33). The serological differences between the two K^b subsets could suggest that certain endogenous

peptides may be predominantly bound to one or another K^b subset. Despite the observed serological differences, relevant peptides could be extracted from both K^b subsets with comparable yield for sensitizing T2K^b cells for alloantigen-specific CTL lysis. These results indicate that the functional differences between the two K^b subsets are not likely to be due to the absence or insufficiency of the relevant peptide(s) bound with K^b.

Given that both K^b subsets contain sufficient amounts of relevant peptides for allo-CTL stimulation, we considered a second possibility for why the Ly-m11$^+$ K^b subset did not trigger the CTL clones; namely the Ly-m11$^+$ K^b subset contains peptide-K^b complexes that are TCR antagonists, similar to what has been reported for complexes of MHC molecules and certain synthetic peptide antigen analogues (42, 43). However, our results indicate that the Ly-m11$^+$ K^b subset is unable to inhibit the binding and response of the allo-specific CTL clones when coimmobilized with stimulatory M1/42^{high} K^b subset molecules, suggesting that the Ly-m11$^+$ K^b subset does not apparently contain strongly antagonistic activities.

We observed a significant difference in molecular mass of the two K^b subsets, with the M1/42^{high} K^b subset being ~47 kD and the Ly-m11$^+$ K^b subset ranging from 51–56 kD. Murine class I at the higher molecular mass has been occasionally observed before in immunoprecipitates (7, 44). When present in immunoprecipitates, the higher molecular mass class I is sometimes assumed to represent class I heavy chain with β_2m still associated. However, we consider this possibility for the isolated Ly-m11$^+$ K^b subset very unlikely since there was no change in the migration of this subset in gels run in the presence of 8 M urea and 100 mM dithiothreitol (data not shown). Since the pattern of mobility of the Ly-m11$^+$ K^b subset is diffuse, this more likely suggests that differences in glycosylation may be contributing to the observed heterogeneity between the two isolated K^b subsets. Consistent with this possibility, we found that upon Endo F treatment to remove N-linked carbohydrate structures, both K^b subsets migrated coincidently. Murine class I molecules have two or three N-linked glycans with K^b usually possessing two N-linked complex-type glycosylations at Asn-86 and Asn-176 of the α_1 and α_2 domains, respectively (45). Microheterogeneity of murine class I molecules has been observed previously and has been attributed to differences in the carbohydrate structures attached to the heavy chain (6–8). Often two major subsets that differ in size as well as charge can be observed by two-dimensional gels from immunoprecipitations of some H-2 class I molecules (6, 7). In some cases the decreased mobility and higher negative charge of one subset can be attributed at least in part to greater sialylation (6–8).

Despite the fact that class I carbohydrate microheterogeneity has been shown to exist, we demonstrate in this report that at least two subsets of cell surface expressed K^b, differing in N-linked carbohydrate modification can be independently isolated and analyzed biochemically and functionally. The higher molecular mass of the Ly-m11$^+$ K^b may possibly be due to differences in its degree of sialylation relative to the M1/42^{high} K^b subset. However, the relative resistance of the Ly-m11$^+$ K^b to NANAse treatment suggests either that sialy-

lation is not the major contributor to the reduced mobility or that sialylation may be responsible, but most of the sialic acid linkages are of a type that is resistant to routine NANAse treatment (41). An alternative or additional possibility is that the hyperglycosylated Ly-m11$^+$ K^b may be modified by poly-N-acetyllactosamine (PL) addition(s) similar to ABO blood group antigens that can be found on RBC and hematopoietic cells (46). Multiple and variable lactosaminyl unit additions can result in a mixture of multiple sizes of a glycoprotein (46), observed as broadening and smearing in SDS-PAGE, similar to what we observe for the Ly-m11$^+$ K^b subset.

It has been observed previously that the N-linked carbohydrate on murine class I molecules may affect their recognition by some monoclonal antibodies (9, 47). It is presently unclear why the Ly-m11$^+$ K^b subset is more readily eluted from the Y3 column than the M1/42^{high} K^b subset. However, it is conceivable that the affinity of the Y3 mAb for the Ly-m11$^+$ subset may be diminished due to the apparent greater size of the carbohydrate side chains on the Ly-m11$^+$ K^b subset. It is possible for the same reason, that the larger carbohydrate found on the Ly-m11$^+$ K^b subset negatively affects M1/42 mAb recognition possibly due to steric hindrance, masking of an epitope or it lacks a specific carbohydrate structure that may be involved in M1/42 recognition of class I. Recognition of K^b complexes by the S19.8 (Ly-m11) mAb may be enhanced by certain carbohydrate structures found on one, but not the other K^b (M1/42^{high}) subset. Further studies will be required to determine the influence of N-linked carbohydrate structure on M1/42 and S19.8 antibody recognition of K^b molecules.

It has been reported previously that N-linked carbohydrates are not required for CTL recognition (9, 10). Removal of N-linked carbohydrate from K^k by Endo F treatment does not affect allo-specific CTL proliferation (10), and amino acid substitutions at all three Asn carbohydrate attachment sites on L^d by site-directed mutagenesis also did not result in a significant change in allo-CTL lysis of cells transfected with the mutated L^d gene (9). Despite observations indicating that the N-linked carbohydrates on class I seem not to be required for CTL recognition, the effects of the extent and composition of the class I carbohydrate side chains on CD8$^+$ CTL recognition is not understood. Our data suggest for the first time that the type of N-linked carbohydrate on class I is likely to affect TCR recognition of class I alloantigen. How might the carbohydrate expressed on the Ly-m11$^+$ K^b subset negatively affect TCR recognition? Several explanations are possible including: (a) because of their larger size, the carbohydrate structures on the Ly-m11$^+$ K^b subset may sterically block TCR interaction(s); (b) the Ly-m11$^+$ K^b subset expresses carbohydrate with a high net negative charge due to a high degree of NANAse resistant sialylation or possibly sulfation, resulting in repulsion between the TCR and class I; (c) the carbohydrate on the two K^b subsets may affect the conformation of the heavy chain differentially; subtle changes in class I conformation are known to affect TCR recognition (36, 48); (d) some combination of the above possibilities. We have treated the Ly-m11$^+$ K^b subset with Endo F or NANAse to remove N-linked glycosylation or its ter-

minal sialic acids respectively, in order to convert the Ly-m11$^+$ K^b subset to a functional TCR ligand, however with current methods the K^b in both subsets is not sufficiently stable during enzyme treatments to assay for CTL recognition (data not shown).

The Ly-m11 epitope has been detected on the surface of a variety of normal and transformed cell types (18, 22). Almost all spleen and lymph node cells and a portion of bone marrow and thymus cells express this K^b and β_2m-dependent epitope. Tumor cells derived from various hematopoietic cell types from C57BL/6 and related strains are also positive for this antigen. Consistent with these previous observations, we were able to purify Ly-m11$^+$ K^b from a variety of H-2^b expressing tumor cell types of hematopoietic origin. We have also attempted to purify the two K^b subsets from normal spleen and thymus cells, however the yields of K^b were not sufficient to be tested in assays done with both K^b subsets isolated from tumor cells (data not shown). Therefore, a direct comparision of K^b subsets from normal and transformed cells has not been possible and their potential structural and functional relationships remain unresolved.

The two cell surface forms of K^b that differ in their abilities to trigger antigen specific CTL may reflect antigen presentation by class I in vivo. For example, different cells may express varying densities of the two functionally distinct K^b subsets, perhaps as suggested by the differing relative yields of the subsets from different cell lines (Fig. 1 and data not shown), and this might, in certain circumstances, influence their abilities to serve as antigen presenting cells. From our results, peptides load both subsets equally well, yet only the complexes of peptide and M1/42^{high} K^b serve as both a TCR and CD8 ligand and induce a response. If expression of the Ly-m11$^+$ K^b subset is high on a cell, it may compete with the M1/42^{high} subset for binding to a limiting peptide antigen, and thus maintain the density of the relevant ligand for TCR recognition at a level below that for effective response. Further studies are necessary to determine whether this can indeed occur.

Tumor cells, especially those with a highly metastatic phenotype, are known to express altered carbohydrate moieties on some of their glycoproteins (46, 49). It is conceivable that alteration of the carbohydrate structures expressed on tumor cell class I molecules could diminish or prevent its recognition by the TCR, thus providing a means of escaping CD8$^+$ T cell surveillance. One of the modifications resulting from cell transformation is increased addition of PL to N-linked carbohydrate chains (49). Since it is possible that PL may be responsible for the larger carbohydrate found on the Ly-m11$^+$ K^b subset, it may also be responsible for the failure of the K^b subset to serve as a TCR ligand.

Although the carbohydrate on the Ly-m11$^+$ K^b subset might interfere with TCR recognition, it may be necessary for interaction with other class I binding receptors, e.g., Ly-49A and/or related family members (50–52). Ly-49A has recently been demonstrated to be a class I MHC binding receptor normally expressed on a subset of NK cells, that when engaged may negatively regulate NK lytic activity (50–52). The Ly-49 family belongs to the greater C-type lectin family and

a related rat molecule, NKR-P1 specifically expressed on NK cells, has recently been demonstrated to bind carbohydrates (53). It is therefore possible that a specific carbohydrate struc-ture linked to K^b, perhaps as displayed by the Ly-m11$^+$ subset, may be required for recognition by Ly-49 family member(s).

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