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Single-residue changes in class I major histocompatibility complex molecules stimulate responses to self peptides

(alloreactivity/T-cell receptor)

ANDRES G. GRANDEA III AND MICHAEL J. BEVAN*

Howard Hughes Medical Institute, Department of Immunology, University of Washington, SL-15, Seattle, WA 98195

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ABSTRACT **Single-residue changes were introduced into the murine major histocompatibility complex class I molecule H-2K^b at positions 65 and 69, which are predicted to point up from the α-helix of the α_1 domain and not into the peptide binding groove. Mutated and wild-type genes were transfected into the murine cell line P815 (*H-2^d*). We present evidence that the changes did not affect the binding of three foreign peptides that are recognized by cytotoxic T lymphocytes (CTL) in association with H-2K^b. Additionally, the mutants provoked strong alloreactive responses in T cells from mice expressing unmutated H-2K^b. The alloreactive CTL were specific for self peptides, which could be extracted from wild-type H-2K^b molecules, recognized in the context of the mutant class I.**

The response of T lymphocytes to foreign antigens is controlled by cell-surface glycoproteins encoded in the major histocompatibility complex (MHC). The class I and class II MHC molecules present short peptide fragments of foreign antigens to CD4$^+$ helper T lymphocytes and CD8$^+$ cytotoxic T lymphocytes (CTL), respectively (1, 2). Recent work has shown that short octamer or nonamer peptide fragments are bound by the class I MHC molecule (3–5). The highly polymorphic residues within the class I peptide binding groove determine which peptides will be bound (6). It is not known with certainty what parts of this peptide–MHC complex are actually contacted by the T-cell receptor (TCR). In the models that explain TCR contact (7–10), the selection of peptides brought to the surface by the MHC and the position of these peptides within the groove can explain the phenomenon of MHC restriction.

A large fraction of T cells respond to foreign MHC molecules. The currently popular explanation for this alloreactive response is that a foreign MHC molecule will bind different self peptides from that bound by self MHC molecules, resulting in a large number of new peptide–MHC complexes displayed on the surface. A form of this model was originally suggested in 1977 (11) and has been supported in that many alloreactive T cells are, in fact, specific for the foreign MHC molecule plus a particular peptide. The work of Sherman and collaborators has shown that many alloreactive CTL clones recognize foreign class I molecules when artificially produced peptides derived from cellular proteins are added to target cells (12, 13). Different alloreactive clones are dependent on different peptides (14). This model probably explains the alloreactive response of *H-2K^b* mice to cells expressing mutant H-2K^b molecules in which the changes point into the peptide binding groove (15). Another explanation for the high frequency of T-cell responsiveness to a cell bearing a nonself MHC molecule is that certain parts of the foreign MHC molecule that are not affected by the particular peptide bound will present foreign determinants at a much higher density than any determinant that depends on the complex of peptide plus MHC residues for recognition (16). In support of this view, recent evidence suggests that purified HLA-A2 molecules devoid of peptides can stimulate alloreactive CTL (17).

We made single-residue changes in the H-2K^b molecule at positions 65 and 69 that are predicted to point up from the α-helix of the α_1 domain and therefore should not affect the specificity of the peptide binding groove (6, 18). In support of this, we present evidence that the mutant molecules bind the same range of foreign and self peptides as the wild-type H-2K^b molecule. All of these single-residue changes were able to stimulate a strong, alloreactive response in cells from mice that themselves express the wild-type H-2K^b molecule. By extracting naturally processed peptides from H-2K^b wild-type molecules, we show that the mutant-specific alloreactive T cells are, in fact, specific for a self peptide (i.e., one that is also presented by their own wild-type H-2K^b molecule) plus the single change in the MHC-presenting molecule.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) and (C57BL/6 × DBA/2)F$_1$ (BDF$_1$) mice were purchased from The Jackson Laboratory.

Peptides. Ovalbumin (Ova) fragment from position 242 through 276 [Ova-(242–276)] (19) was obtained from either the peptide synthesis facility at Scripps Clinic and Research Foundation or from the Howard Hughes Medical Institute Organic Synthesis Facility at the University of Washington. Sendai virus nucleoprotein (NP) fragment NP-(321–332) (20) and vesicular stomatitis versus (VSV) nucleocapsid (N) protein fragment N-(53–63) (4) were obtained from the latter source.

Site-Directed Mutagenesis. The *H-2K^b* variant DNA constructs were made by oligonucleotide-directed mutagenesis of a 0.7-kilobase (kb) *Kpn* I fragment subcloned into the single-stranded phage M13mp8 as described by the provider of the mutagenesis kit (Amersham). The resulting mutated *Kpn* I fragments were either cloned into the parental plasmid pH-2K^b, which carries an 11-kb genomic fragment encoding H-2K^b (21), or the plasmid pK^{b}pTCF (obtained from K. Rock, Harvard Medical School), which carries the same 11-kb fragment and the bacterial neomycin-resistance gene (22). The plasmid encoding glutamic acid at position 65 is a mutated form of pH2-K^b, and the four other constructs are mutations of pK^{b}pTCF.

Transfections. The DBA/2-derived mastocytoma P815 cell line was electroporated with each of the pH2-K^b-derived constructs along with pSV2-neo or with each of the pK^{b}pTCF-derived constructs. The B6-derived tumor cell RMA-S (23) was electroporated with the pK^{b}pTCF/D65 construct.

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Abbreviations: MHC, major histocompatibility complex; CTL, cytotoxic T lymphocytes; TCR, T-cell receptor; Ova, ovalbumin; VSV, vesicular stomatitis virus; N, nucleocapsid; NP, nucleoprotein; E:T, effector-to-target cell ratio.

*To whom reprint requests should be addressed.

Isolates were selected with RP10 [RPMI 1640 supplemented with 10% (vol/vol) fetal bovine serum] containing G418 at 0.6 mg/ml and were maintained in RP10 containing G418 at 0.4 mg/ml.

The wild-type H-2K^b molecule has glutamine at position 65 and glycine at position 69. The mutant with Asp-65 is named D65; with Glu-65, E65; with Arg-65, R65; and with Val-65, V65. The mutant with Val-69 is named V69.

Identification of Mutant H-2K^b Expressors. The isolates of P815/H-2K^b or P815/H-2K^b-mutant transfectants were stained with the anti-H-2K^b monoclonal antibody Y3 (a 1:200 dilution of ascitic fluid) (24), followed with a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin and subjected to fluorescence-activated cell sorting analysis. High expressors of the wild-type or mutant H-2K^b molecules were used for further analyses.

Ova-Specific CTL Clones. The isolation and maintenance of these cells have been described (25).

Alloreactive CTL. For alloreactive lines, spleen cells from unimmunized BDF$_1$ mice were stimulated once *in vitro* with each of the P815/H-2K^b mutant cell lines. In 20 ml of RP10, 4×10^7 responder cells were mixed with 4×10^6 irradiated (3×10^4 R) stimulator cells. Surviving effector cells were used 5 days later in a ^{51}Cr-release assay.

Alloreactive CTL clones specific for the H-2K^b *D65* mutant molecule were isolated by limiting dilution of BDF$_1$ cell lines stimulated *in vitro* with the P815/D65 transfectant.

Extraction and Chromatography of H-2K^b-Bound Peptide. Two B6 spleens were subjected to CF_3COOH extraction and HPLC analysis by following the protocol of Falk *et al.* (26). Individual fractions collected from the reverse-phase HPLC column (Delta Pak C_{18}-300A from Waters) were lyophilized and resuspended in 1 ml of RPMI 1640; 50 μl of each fraction was assayed for sensitization of labeled target cells to lysis by CTL.

For the isolation of H-2K^b molecules, a cell suspension from five B6 spleens was made in phosphate-buffered saline (PBS) and placed in 7 ml of PBS containing 0.5% Nonidet P-40, 10 μM iodoacetamide, and 1 mM phenylmethylsulfonyl fluoride at 4°C for 30 min. The lysate was cleared of insoluble material, and 0.3 ml of protein A-Sepharose and mouse IgG at 100 μg/ml were added to the supernatant. The mixture was incubated at 4°C for 4 hr, and the Sepharose was collected by centrifugation. Ascites (0.1 ml) containing the monoclonal antibody 28-14-8S (27), which binds H-2D^b, and 0.3 ml of protein A-Sepharose were added to the supernatant and incubated at 4°C for 4 hr. The Sepharose was collected as before. Finally, 0.1 ml of the anti-H-2K^b Y3 ascites was added to the same supernatant, which was treated similarly.

The Sepharose beads containing either the precipitated H-2D^b molecules or the H-2K^b molecules were each extracted in 2 ml of 0.1% CF_3COOH for 30 min at 4°C and rinsed with an additional 1 ml of 0.1% CF_3COOH. The two resulting CF_3COOH extracts were lyophilized and resuspended in 0.5 ml of 0.1% CF_3COOH. Low molecular weight material from these extracts was filtered through Centricon 10 microconcentrators (Amicon), subjected to HPLC fractionation, and assayed for targeting activity.

^{51}Cr Release Assays. For the Ova peptide assays, 0.1 mCi (1 Ci = 37 GBq) of sodium [^{51}Cr]chromate (0.1 ml) was added to 10^6 target cells in 0.1 ml of RP10. The cells were washed twice in PBS and then resuspended to 10^5 cells per ml in RP10; 10^4 cells were added to effector CTL in 96-well plates with or without the Ova-(242–276) fragment. Supernatants were collected from the wells after a 3-hr incubation at 37°C and assayed for radioactivity. Controls without Ova-(242–276) exhibited 0–5% specific lysis (unpublished data).

For the peptide competition assay, Sendai virus NP-(321–332) and VSV N-(53–63) were added at the concentrations indicated in Fig. 2 with 10 μM Ova-(242–276) to ^{51}Cr-labeled target cells. The assay was done as before with CTL clones GA4 and HB16 as effectors. The peptides were included throughout the 3-hr incubation of the assay.

The alloreactive BDF$_1$ CTL lines were assayed on day 5 after a single *in vitro* stimulation with each of four mutant H-2K^b-expressing P815 transfectants.

To test for targeting activity in the CF_3COOH extracts, the target cell lines RMA-S and RMA-S/D65 were incubated overnight and labeled with ^{51}Cr at 31°C. A 50-μl aliquot from each HPLC fraction was added to 10^4 target cells in one well of a 96-well plate. The plates were incubated at 31°C for 1 hr. The CTL clones were then added, and the assay proceeded for 6 hr at 37°C.

RESULTS

Changes were introduced into the *H-2K^b* gene to replace amino acid residues 65 or 69. Unmutated *H-2K^b* genes and single-residue variants were transfected into the murine cell line P815 (DBA/2, *H-2^d*), and G418-resistant clones were selected. Transfectants expressing wild-type or mutated H-2K^b molecules were detected with Y3. Additional staining was done with the H-2K^b-specific monoclonal antibodies Y25, EH 144, and 5F1-2-14 (28, 29). There was no variant-specific decline in reactivity with any of these monoclonal antibodies (unpublished data). We conclude that the residue changes do not grossly affect the molecule.

There are three known H-2K^b-restricted foreign peptide epitopes that induce CTL. These are derived from chicken Ova, VSV N protein, and Sendai virus NP. We used these three peptide epitopes to examine peptide binding by the variants. Fig. 1 shows an analysis with four independent H-2K^b-restricted anti-Ova CTL clones. Each one of the H-2K^b variant molecules could present the Ova peptide for

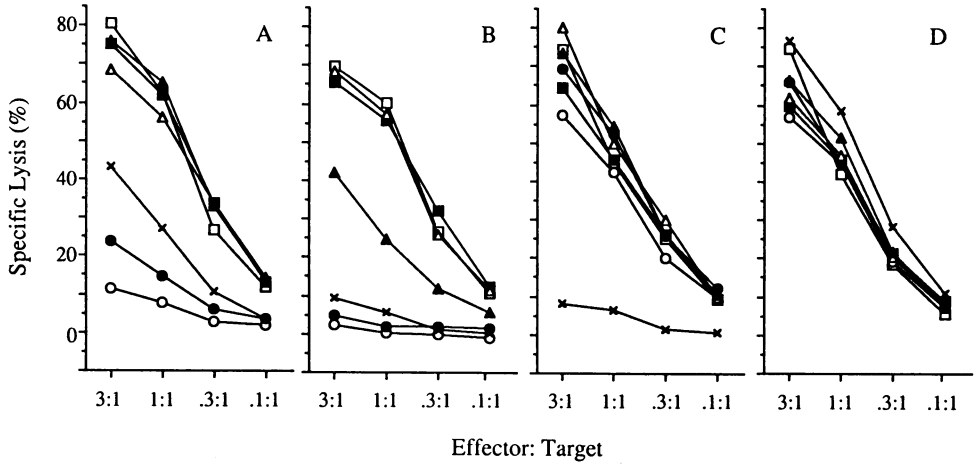

FIG. 1. Presentation of Ova-(242–276) to four CTL clones specific for Ova-plus-H-2K^b by P815 transfectants. CTL clones B3 (*A*), C6 (*B*), GA4 (*C*), and HB16 (*D*) were assayed for lysis of cells expressing wild-type H-2K^b, P815/pH2-K^b (□), and P815/H-2K^b (pTCF) (■), and single-residue variants D65 (○), E65 (●), R65 (△), V65 (▲), and V69 (×) in the presence of 10 μM Ova-(242–276). Controls without peptide gave 0–5% lysis.

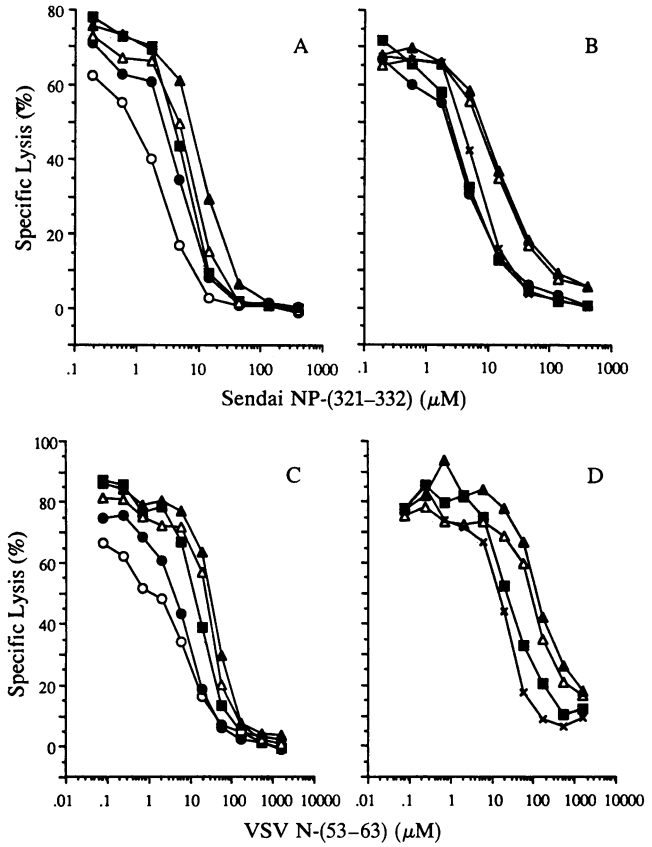

FIG. 2. Competition assay to show that two other peptides bind to the variant H-2K^b molecules. Sendai NP-(321–332) (*A* and *B*) and VSV N-(53–63) (*C* and *D*) peptides were added at the indicated concentrations with 10 μM Ova-(242–276) to ^{51}Cr-labeled P815 transfectants H-2K^b (pTCF) (■), D65 (○), E65 (●), R65 (△), V65 (▲), and V69 (×). The Ova-specific CTL clones GA4 (*A* and *C*) and HB16 (*B* and *D*) were used at an E:T of 10:1.

recognition by at least some of the CTL clones. None of the H-2K^b variant molecules disrupted recognition by all of the CTL clones. Therefore, it is apparent that all five H-2K^b variants can bind and present the Ova peptide. However, individual CTL clones may fail to recognize Ova presented by certain of the variant H-2K^b molecules. For example, CTL clone GA4 failed to recognize Ova with the V69 variant, and CTL clone C6 failed to recognize Ova in association with the V69, D65, or E65 mutants. The likely interpretation of this is that these CTL clones have contact points in this area during their interaction with the class I–peptide complex (28).

The anti-Ova CTL clones GA4 and HB16 were used to study the binding of the Sendai NP and VSV N peptides by the H-2K^b variants in a peptide competition assay. In this assay, the ratio of CTL to labeled transfected targets and the concentration of the targeting Ova peptide were kept constant while the concentration of a competing peptide, Sendai NP-(321–332) or VSV N-(53–63), was varied. The results (Fig. 2) show clearly that both of these viral peptides disrupted Ova-specific lysis of all of the transfected target cells and that in each case the amount of competing peptide needed for 50% inhibition was roughly equal. We conclude that all of the variant H-2K^b molecules can bind these two competing peptides of viral origin.

Each of the single-residue variants could provoke an alloreactive response in spleen cells from unimmunized BDF$_1$ mice. Fig. 3 shows the results with four of the transfectants. CTL induced against the V69 mutant transfectant lysed the homologous target efficiently with little cross-reactivity on the other variants. In addition, CTL raised against R65 cross-reacted to some degree on V65 targets and vice versa. Similarly, the E65 and D65 variants stimulated mutually cross-reactive responses (Table 1 and unpublished data). The P815 transfectants expressing wild-type H-2K^b did not serve as stimulators or targets for this response.

Stimulation of BDF$_1$ spleen cells with P815/D65 also stimulated a strong primary CTL response (unpublished data). To further analyze the specificity of the alloreactive CTL, clones were isolated by limiting dilution from the anti-D65 cultures. Three independent CTL clones were isolated, and their specificity is illustrated in Table 1. They lyse P815/D65 targets extremely efficiently at low effector-to-target (E:T) ratios with little lysis on P815/H-2K^b. One of the CTL clones, d1.2, was cross-reactive on P815/E65 targets.

To investigate the involvement of self peptides or the determinant density required for recognition by these CTL clones, we transfected the D65 variant gene into the class I mutant cell line RMA-S. This cell line has functional class I and β_2-microglobulin genes and yet expresses <5% of the normal amount of class I molecules on the cell surface (23, 30). There is evidence that this cell line is deficient in providing cytosolic peptides to nascent class I molecules in the endoplasmic reticulum (31, 32). The RMA-S cell line can be induced to express much higher levels of H-2K^b and H-2D^b on its surface when cultured overnight at low temperature, 26°–31°C (33). Many of the class I molecules that come to the surface in the cold seem to lack stably bound endogenous peptides. But these empty molecules can be loaded very

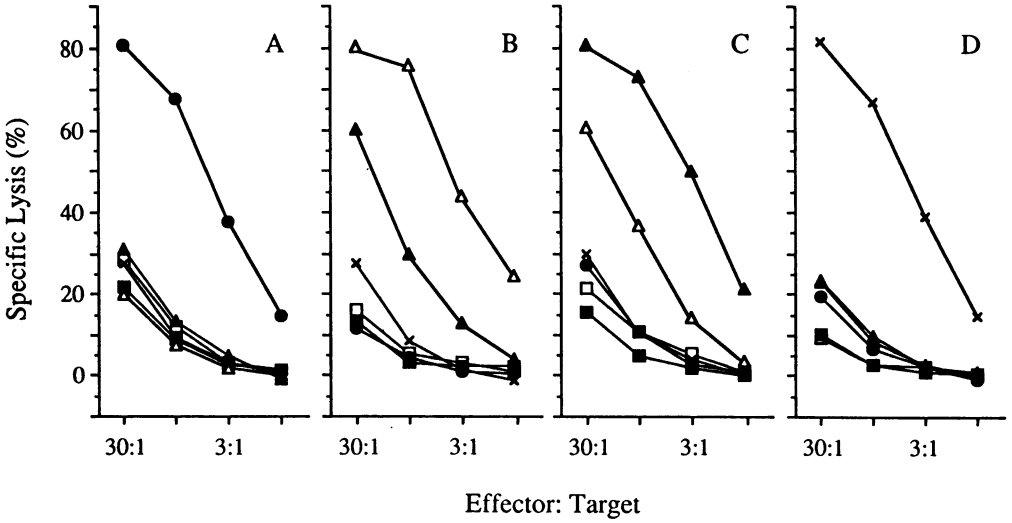

FIG. 3. The mutant H-2K^b molecules provoke an alloreactive response. Spleen cells from unimmunized (B6 × DBA/2)F$_1$ mice were stimulated for 5 days *in vitro* with E65 (*A*), R65 (*B*), V65 (*C*), or V69 (*D*) and were assayed on the P815 transfectants pH-2K^b (□), H-2K^b (pTCF) (■), E65 (●), R65 (△), V65 (▲), and V69 (×).

Table 1. Specificity of three CTL clones derived from BDF_1 splenocytes stimulated with P815/D65 cells

CTL clone	% specific lysis of target cells		
	P815/pH-2K^b	P815/D65	P815/E65
d1.2	13/4	56/27	65/36
d3.1	12/4	45/18	8/4
d3.3	19/11	71/37	17/5

E:T cell ratios in the 4-hr ^{51}Cr-release assay were 0.1:1 and 0.03:1.

efficiently when exogenous peptides are provided in the medium (34). Thus, this RMA-S cell line expressing D65 offered both a way of regulating the level of expression of the D65 molecule on the surface and a sensitive indicator cell to ask if endogenous peptides were required for CTL recognition.

The work of Rammensee and colleagues has shown that naturally processed viral or minor histocompatibility peptides can be extracted from the grooves of MHC class I molecules by CF_3COOH, separated by HPLC, and assayed

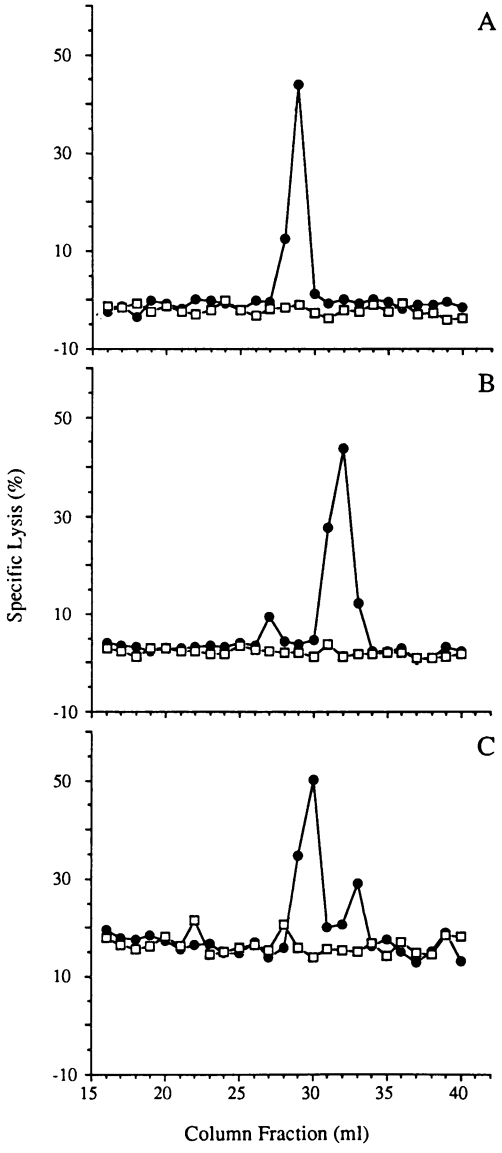

FIG. 4. Acid extracts of C57BL/6 spleen contain targeting peptides for BDF_1 anti-P815/D65 CTL clones. CF_3COOH extracts of C57BL/6 (●) and DBA/2 (□) spleens were fractionated on HPLC and assayed for the ability to enhance the lysis of ^{51}Cr-labeled RMA-S/D65 targets by CTL clone d1.2 at an E:T of 5.1 (*A*), clone d3.1 at an E:T of 9.1, and clone d3.3 at an E:T of 2:1 (*C*).

Table 2. Targeting peptides extracted from transfected P815 cells

Labeled target cell	CF_3COOH extract of cell lines			
	P815	P815/K^b(pTCF)	P815/D65	P815/V69
RMA-S/D65	3/3/4	7/56/3	3/54/5	2/40/4
RMA-S	1/2/1	1/5/1	1/0/1	−1/−1/0

HPLC fractions 28/29/30 from the CF_3COOH extracts of the four indicated cell lines were assayed for their ability to target lysis of ^{51}Cr-labeled RMA-S/D65 or RMA-S cells by CTL clone d1.2 at an E:T of 10:1.

for their ability to target cells for CTL-mediated lysis (3, 26). We applied this technique to detect peptides that would enhance recognition of cold-induced RMA-S/D65 by these three CTL clones. CF_3COOH extracts of splenocytes from B6 mice yielded discrete peaks of targeting activity for the three alloreactive CTL clones, while similar extracts of DBA/2 spleens were negative (Fig. 4). CTL clones d1.2 and d3.1 were specific for peptides that were eluted in HPLC fractions 28/29 and 32, respectively. CTL clone d3.3, which has a higher background level of lysis on the cold-induced RMA-S/D65 targets, was clearly enhanced in its lysis by material in fraction 30.

Specific targeting activity can also be detected in HPLC-fractionated extracts of the transfected tumor cells. Thus, while an extract of wild-type P815 (*H-2^d*) cells yielded no activity that targets the CTL clone d1.2, extracts from the wild-type H-2K^b D65 or V69 transfected P815 cells yielded targeting activity in fraction 29 of HPLC (Table 2). The activity was eluted in the same position from the three transfected cell lines and from C57BL/6 spleen cells. There was no indication that wild-type and variant H-2K^b transfectants differed qualitatively or quantitatively as regards this targeting activity. Fraction 29, which enhanced lysis of RMA-S/D65 targets by CTL clone d1.2, did not increase lysis of RMA-S targets expressing wild-type H-2K^b.

Further experiments were done to establish definitively the source of the targeting peptides and to show that the D65 variant is the restriction element recognized on the target cell. Detergent lysates of B6 spleen cells were subjected to successive rounds of immunoprecipitation with control, anti-H-2D^b, and anti-H-2K^b antibodies. The anti-H-2D^b and anti-H-2K^b immunoprecipitates were extracted with CF_3COOH, and the extracts were fractionated by HPLC (Fig. 5). Clear peaks of targeting activity were observed in the extracts of the anti-H-2K^b immunoprecipitate. The major peak of targeting activity was different for each of the three CTL clones and, in each case, coincided in elution with the peak observed in total CF_3COOH extracts (Fig. 4). Material eluted from the anti-H-2D^b precipitates did not yield targeting activity. The data in Fig. 5 also show that D65 is the restricting molecule on the target cell, since nontransfected RMA-S (H-2K^b, H-2D^b) cells are not lysed.

DISCUSSION

We have presented evidence that five single-residue variants of the H-2K^b molecule (E65, R65, D65, V65, and V69) are able to bind three foreign peptide epitopes recognized by CTL in association with H-2K^b. In addition, the D65 variant can also bind and present a number of self peptides extracted from wild-type H-2K^b cells. It seems likely from these data and from modeling studies based on crystal structures of HLA molecules (refs. 18 and 35; S. Joyce, T. Garrett, and S. Nathenson, personal communication) that the peptide binding grooves of these variants are the same as that in wild type. The same range of peptides is presented, and they may be held in the same conformation in the groove. The T cells from *H-2^b* mice that respond to D65 must be contacting more than just sites on the peptide, as has been suggested (10). All three

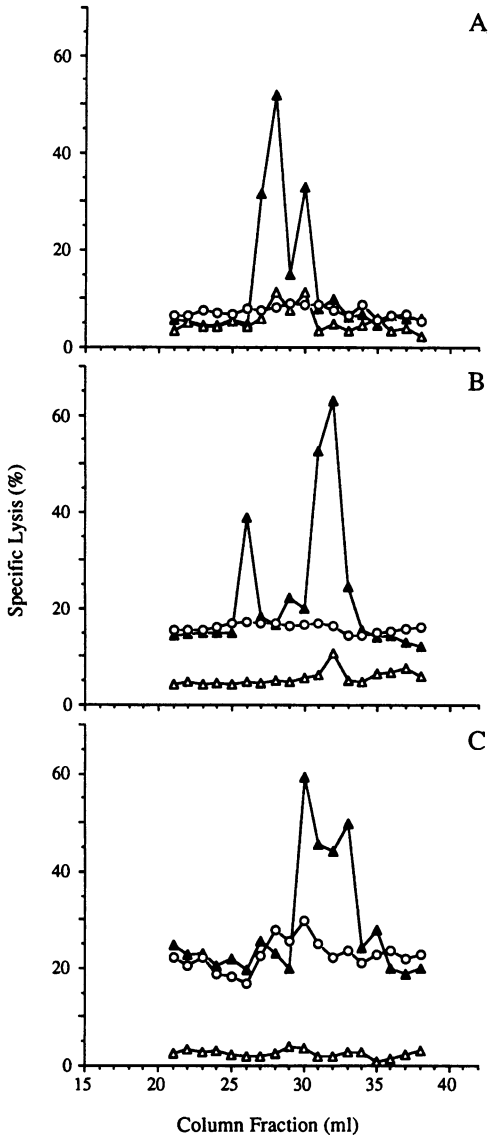

FIG. 5. Acid extracts of immunoprecipitated H-2K^b molecules from C57BL/6 spleen contain targeting peptides for BDF$_1$ anti-P815/D65 CTL clones. CTL clone d1.2 at an E:T of 10:1 (*A*), clone d3.1 at an E:T of 10:1 (*B*), and clone d3.3 at an E:T of 2:1 (*C*) were assayed for lysis of RMA-S/D65 targets (▲ and ○) or RMA-S targets (△) in the presence of HPLC-fractionated extracts of H-2K^b immunoprecipitate (▲ and △) or H-2D^b immunoprecipitate (○).

of the BDF$_1$ anti-D65 CTL clones recognized the D65 variant in association with a specific peptide that could be extracted from H-2K^b molecules but not from D^b molecules or DBA/2 (*H-2^d*) spleen cells. These alloreactive T cells have broken tolerance to a self peptide by seeing it in a new context, namely the change at residue 65.

Our results on the peptide dependency of the anti-D65 CTL provide a clear molecular illustration of the fact that tolerance to self is MHC-restricted (36–38). They support the interpretation of alloreactivity that proposes that alloreactive T cells are specific for MHC plus a peptide. In conventional alloreactive responses, where the foreign MHC would have a different peptide binding specificity, a new range of peptides will be seen in association with the MHC molecule. In the case we have analyzed, BDF$_1$ anti-D65, where the change may not affect the peptide binding groove, we propose that a set of self peptides is recognized in conjunction with the change in the MHC α-helix.

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