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The H4^b Minor Histocompatibility Antigen Is Caused by a Combination of Genetically Determined and Posttranslational Modifications[1]

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Minor histocompatibility (H) Ag disparities result in graft-vs-host disease and chronic solid allograft rejection in MHC-identical donor-recipient combinations. Minor H Ags are self protein-derived peptides presented by MHC class I molecules. Most arise as a consequence of allelic variation in the bound peptide (p) that results in TCR recognizing the p/MHC as foreign. We used a combinational peptide screening approach to identify the immune dominant H2K^b-restricted epitope defining the mouse H4^b minor H Ag. H4^b is a consequence of a P3 threonine to isoleucine change in the MHC-bound peptide derived from epithelial membrane protein-3. This allelic variation also leads to phosphorylation of the H4^b but not the H4^a epitope. Further, ex vivo CD8$^+$ T lymphocytes bind phosphorylated Ag tetramers with high efficiency. Although we document the above process in the minor H Ag system, posttranslational modifications made possible by subtle amino acid changes could also contribute to immunogenicity and immune dominance in tumor immunotherapeutic settings. ***The Journal of Immunology*****, 2003, 170: 5133–5142.**

Tissue transplantation is a major therapeutic modality for end stage disease of affected organs. Despite matching of donor and recipient *Mhc*-encoded class I and class II molecules, rejection of the allografts ensues. Rejection of MHC-matched allografts results from T lymphocyte responses focused on non-*Mhc*-encoded alloantigens called minor histocompatibility (H)[4] Ags. Minor H Ag incompatibility most impacts clinical bone marrow transplant recipients in whom graft-vs-host disease could result in fatality. Thus, understanding the molecular basis for minor H Ag presentation and recognition is of clinical import.

More than a decade ago, the pioneering work of Rammensee (1, 2) and Fischer Lindahl (3, 4) and their coworkers independently recognized that minor H Ags are self peptides, derived from proteolytic processing of normal cellular proteins. These self peptides are presented by MHC class I molecules to specific CD8$^+$ T lymphocytes. Positional cloning, cDNA expression cloning, and biochemical (reversed phase chromatographic separation of the epitope isolated from the class I molecule and amino acid sequence determination by Edman degradation or mass spectrometry) approaches have led to the identification of several human and mouse minor H Ag-derived CTL epitopes (reviewed in Ref. 5). The data thus far indicate that immune responses to minor H Ags could result from the recipient carrying a null allele (e.g., H60 and HY male Ag) (6, 7) or differential or induced expression (e.g., H28) (8), differential processing (e.g., human minor H Ag HA8) (9), or amino acid sequence variation in the peptide epitope (e.g., H3 and H13) (10, 11). Sequence variation is a consequence of evolving genomes. Variant peptides, when presented by the donor class I molecule that is identical with that of the recipient, appear nonself to the host. Differences as subtle as a loss of a methylene group in a TCR contact residue are sufficient to elicit a CTL response (10, 12), which then culminates in allograft rejection. Thus, minor H Ags are highly immunogenic.

We have adopted a mouse model, which involves immunization of C57BL/6 mice with H2^b-identical BALB.B alloantigens encoded by multiple *minor H* gene loci (13, 14). This model closely recapitulates MHC-identical clinical bone marrow transplantation wherein graft-vs-host response evolves due to minor H incompatibility between the donor and the recipient. Whereas C57BL/6 and BALB.B differ at numerous *minor H* loci, the evolving CTL response is focused on select minor H Ags (13, 14). For example, C57BL/6 CTL response to BALB.B minor H Ags is focused primarily on H2K^b-restricted H60, but responses to H2K^b-restricted H4 and H28 as well as to H2D^b-restricted H7, H13, and HY alloantigens are also observed. The magnitude of the response to H60 is greater than those toward the other minor H Ags (15–17). This hierarchic T cell response, also observed in immunity to pathogens and tumors, is termed immune dominance (reviewed in Ref. 18). Despite its relevance, the molecular basis of immune dominance to CTL Ags is poorly understood.

All the BALB.B alloantigens recognized by C57BL/6 CTL but H4 have been identified and characterized. Under certain circumstances, B10.129-*H4^b* congenic male skin graft or bone marrow recipient C57BL/6 (H4^a; hence H4 disparate) mice elicit a dominant CTL response to the H4^b alloantigen, thus dominating the male HY minor H Ag (19). Therefore, to establish a simple system

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[4] Abbreviations used in this paper: H, histocompatibility; RP, reversed phase; TFA, trifluoroacetic acid; MFI, mean fluorescence intensity.

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to study the basis of immune dominance, we herein report the biochemical characterization of the H2K^b-restricted H4^a- and H4^b-specific CTL epitopes. Characterization of the H4^b epitope led to the identification of the gene encoding this epitope and determination of its immunogenicity in the recipient.

Materials and Methods

Animals

C57BL/6J, 129/SvJ, C.B10-*H2^b*LilMcdJ (BALB.B), B6.C-*H60^c*/DCR and B10.129-*H46^{b}H47^b*(21 M)/Sn mice were obtained from The Jackson Laboratory (Bar Harbor, ME). They were bred and maintained in the Joyce and Roopenian mouse colonies in accordance with the Institutional Animal Care and Use Committee policies of Vanderbilt University and The Jackson Laboratory, respectively.

Cell lines

RMA, RMA-S (20), B6/wt19 (21) (a gift from S. S. Tevethia, Hershey Medical Center, Pennsylvania State University, Hershey, PA), Jurkat and Jurkat-K^b (Ref. 22; a gift from L. A. Sherman, The Scripps Research Institute, San Diego, CA) were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 5–10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, 100 μg of streptomycin, and 100 μM L-glutamine. NS0, a H2^d plasmacytoma, was maintained in DMEM (Life Technologies) supplemented with FCS, penicillin, streptomycin, and L-glutamine. Kb-high NS0 transfected with full length H2K^b cDNA was maintained in L-glutamine-deficient DMEM (Life Technologies) supplemented with 5–10% dialyzed FCS (HyClone), penicillin, streptomycin, essential amino acids, and nucleosides as described (23).

Peptide synthesis

Peptides were synthesized by Fmoc chemistry in either a micromole scale using pin technology (Chiron Mimotopes, Emeryville, CA) or a millimole scale (Pennsylvania State University College of Medicine) as described previously (15, 25, 26). Phosphopeptides were also synthesized by Fmoc chemistry using phosphoserine, phosphothreonine, or phosphotyrosine during coupling. Peptides were provided >90% pure as judged by reversed phase (RP) chromatography and/or matrix-assisted laser desorption/ionization or electrospray ionization mass spectrometric analyses (data not shown). Stock solutions of the peptide were prepared in DMSO at ~20 mg/ml. All peptides were diluted in RPMI 1640 containing 5% FCS for the CTL assay. Heat-inactivated FCS has low concentrations of proteases and hence is recommended for use in cell cultures where peptide stability is important (27).

Isolation and characterization of MHC class I-associated peptide epitopes

Peptide isolation and fractionation. Peptides and proteins were directly precipitated from splenocytes with 0.1% v/v trifluoroacetic acid (TFA) as described. After sedimentation of cellular debris by ultracentrifugation at 100,000 $\times g$, peptides in the soluble fraction were separated from proteins by Centricon 3 (Centricon, Bedford, MA) filtration. Peptides in the filtrate were concentrated by vacuum centrifugation, and their concentration estimated by microBCA (Pierce, Rockford, IL) assay. They were separated by microbore RP chromatography using either a C18 or a mixture of C18 and cation exchange column (250 $\times$ 1 mm, 120 Å, 5 μm; Alltech, Deerfield, IL) operated by a 1090 HPLC (Hewlett Packard, Palo Alto, CA). Peptides were eluted using a gradient (gradient 1) of buffer B generated over a period of 125 min at a flow rate of 0.05 ml/min as follows: 0–20% buffer B over 15 min, 20–45% buffer B by 95 min, and 45–100% buffer B by 125 min. The composition of buffer A was 0.06% TFA in water (Burdick and Jackson, Muskegon, MI), whereas the composition of buffer B was 0.056% TFA in acetonitrile (Burdick and Jackson). Peptide elution was monitored at 214 and 280 nm. Two-drop fractions corresponding to a volume of ~30–40 μl were collected. Alternatively, columns were eluted with a gradient (gradient 2) of buffer B built over 110 min at a flow rate of 0.05 ml/min as follows: 0–37% buffer B over 63 min; 37–70% buffer B by 70 min; 70–90% buffer B by 105 min; and 90–100% buffer B by 110 min. Peptide elution was monitored and fractions were collected as described above.

Alkaline phosphatase digestion. In some experiments, TFA extracts were dissolved in an alkaline buffer (50 mM Tris-Cl, pH 8.0) and divided in two equal aliquots. One aliquot was treated with a mixture of serine and threonine phosphatase inhibitors (cocktail 1: cantharidin, bromotetramisole, and microcystin LR; Sigma-Aldrich, St. Louis, MO), and the other aliquot was treated with 10 U alkaline phosphatase (Sigma-Aldrich) according to the manufacturer's instructions. After 1 h at 37°C, the reaction was stopped by dilution in water. Low molecular mass peptides were isolated by Centricon 3 filtration. The filtrate was separated by RP chromatography, and the CTL active fractions were identified by target reconstitution assay.

Target reconstitution. For sensitization of target cells in ^{51}Cr release assays, between 2 and 30 μl of each peptide fraction were used. TFA and acetonitrile contained in the peptide fractions were removed by dilution in water, followed by evaporation under vacuum. Before the assay, 20 μl of concentrated peptide fractions were resuspended in 400 μl of RPMI 1640 supplemented with 5% FCS.

Generation of minor H Ag-specific CTL, CTL clones, and ^{51}Cr release assay

The H2K^b-restricted H4^b-specific CTL were generated by immunization of C57BL/6 mice with 2 $\times$ 10^7 C.B10-*H4^b* (21 M) splenocytes twice at weekly or biweekly intervals. One week after the second immunization, the responders were mixed with 1.25 times the cell density of stimulator 21 M splenocytes irradiated at 6000 rad. Five days later, the expanded T lymphocytes were used in a tetramer binding assay (described below). The H2K^b-restricted H4^b-specific CTL lines M9 and M11 were isolated from C57BL/10 mouse, primed and restimulated with H4^b-congenic 21 M splenocytes, as described (28). Two cloned lines were established and maintained with weekly stimulation using irradiated 21 M splenocytes in the presence of 50 U/ml rIL-2, as described (28). The H2K^b-restricted H4^a-specific CTL line RC6 was generated from 21 M mice immunized with C57BL/6 splenocytes as described for M9 and M11.

A ^{51}Cr release assay was performed according to standard protocols. The E:T ratios for H4^b-specific CTL assays are described in the figure legends. ^{51}Cr-labeled target cells (2 $\times$ 10^5 cells/ml) were preincubated for 2 h with an equal volume of peptide solution in RPMI 1640 supplemented with 5% FCS. After 2 h at 37°C, an equal volume of effector cells were added and incubated at 37°C for 5–6 h in microtiter plates. One-twentieth of the supernatant in each well (~10 μl) was harvested, mixed with Microscint-PS (PerkinElmer, Wellesley, MA) scintillant and monitored for radioactivity using TopCount plate reader (PerkinElmer). Data are represented as percent specific lysis.

tblastn search

SGIVYIHL peptide sequence was used to search for the gene encoding the H4^b epitope within the dynamically translated nucleotide sequence bank at http://www.ncbi.nlm.nih.gov.

PCR cloning and nucleotide sequence determination

Genomic DNAs from C57BL/6 and BALB.B mouse tails were isolated and used as the source of *Epithelial membrane protein-3* (*Emp-3*) gene. Using 5′-GCACTCATCTATGCCATCC-3′ and 5′-TCATTCACGTTTCCGCAG-3′ oligonucleotide primers, exon 5, which encodes the region containing the H4 epitope, was amplified by standard PCR. The 166-bp product was cloned into pCR3.1TOPO PCR cloning/mammalian expression vector (Invitrogen, Carlsbad, CA). Two molecular clones amplified from each mouse strain were subjected to automated dideoxy chain termination sequence analysis (Vanderbilt University DNA Sequencing Facility).

H4 minigene construct and expression

Exon 5 of *H4^a* and *H4^b* was PCR amplified from C57BL/6 and BALB.B genomic DNAs, respectively. The forward primer 5′-GCCATGGCACT CATCTATGCCATCCA-3′ contains the consensus Kozak motif and the initiator ATG (underlined) to ensure expression. The reverse primer 5′-TCATTCACGTTTCCGCAGGT-3 contains the endogenous stop codon (underlined). The amplified minigene was cloned into pCR3.1-TOPO (Invitrogen). One microgram of an authentic *H4^a* and *H4^b* minigene, as judged by nucleotide sequence analysis, was transfected into ~3 $\times$ 10^5 B6/wt19 cells using Fusgene 6 (Invitrogen) according to the manufacturer's instructions. One day later, the transfectants were used as targets in CTL assay.

Preparation and use of class I peptide tetramers

Preparation of H2K^b-peptide and H2D^b-peptide tetramers have been described previously (15, 26). BALB.B-derived minor H Ag immune splenocytes were reacted with H2K^b-H4^b, H2K^b-^{P}H4^b, H2K^b-H60 and H2D^b-HY tetramer-PE in conjunction with anti-CD8a-FITC for 2 h at 37°C. After removal of unbound reagents, cells were fixed and,the staining intensity was measured by flow cytometry.

Table I. *List of peptides used in this study and their physical and biological properties:* first-generation mimotopes

Sequence	Hydrophobic Index[a]	RP Chromatography Retention Time (Gradient[b])	M9 Peptide Concentration at Half-Maximum Lysis ($\times 10^{-12}$ M)[c]
Natural H4[b]	<0.74[d]	55.3/56.0	
EGIIFVHL[e]	0.76	ND/57.9	ND[f]
EGIIFIHI	1.06	ND	20/300
EGIVFIHI	0.99	ND	20/60
EGIYFIHI	0.96	ND	30/300
EGIIFIRI	0.92	ND	1000/150
EGIYFIRI	0.85	ND/60.8	600
EGIVFIRI	0.81	ND/61.4	450
EGIIFIHL	1.05	63.5	15/45
EGIVFIHL	0.98	61.0	40/30
EGIYFIHL	0.94	ND	15/15
EGIIFIRL	0.90	59.0	20/15
EGIVFIRL	0.83	60.0/61.6	20
EGIYFIRL	0.80	ND/62.1	30
EGIIFIHV	0.99	ND	15/60
EGIVFIHV	0.92	ND	10/10
EGIYFIHV	0.88	ND	20
EGIIFIRV	0.85	59.0	20
EGIVFIRV	0.77	57.0/56.6	45/20
EGIYFIRV	0.74	58.0/57.3	6/50

[a] Based on Ref. 47.

[b] RP chromatography conditions used to separate peptides in Tables I and II were different; note that the columns used for chromatography of peptides described in this table and Fig. 2 are different from that used for peptides fractionated in Figs. 3, 5, and 7. See *Materials and Methods* for details. Also, the two numbers in this column correspond to the elution times obtained from two similar RP chromatographies of the same mimotope.

[c] Determined as described in Fig. 2 with H4[b]-specific M9 CTL clone. The two values reflect two independent determinations.

[d] Based on the hydrophobicity of the mimotope that migrates closest to the natural epitope.

[e] Based on Ref. 29.

[f] ND, not determined because the hydrophobic index of these peptides was higher than the natural Ag and/or the biological activity was elicited by very high amounts of the mimotopes.

Peptide binding assay

RMA-S cells (~0.5–1.0 × 10^6) grown for 18–20 h at 26°C were incubated with 10-fold increases, ranging from 10^{-10} to 2 × 10^{-6} M, of test and control peptides. After 45 min at 26°C in the presence of peptides, cells were washed to remove unbound ligands and incubated at 37°C for an additional 4 h in tissue culture medium. After the removal of released peptides, cells were reacted with biotinylated mAb against H2K[b] (AF6-88.5) or H2D[b] (KH95), washed free of unbound mAb, and stained with streptavidin-PE (all from BD PharMingen, San Diego, CA). Fluorescently labeled cells were washed, fixed with 2% v/v paraformaldehyde, and analyzed using FACSCalibur (BD Biosciences, San Jose, CA) and CellQuest version 3.0 (BD Biosciences). Data are presented as percent maximum mean fluorescence intensity (MFI).

The relative affinity (K_d) of peptide class I was calculated from specific MFI (difference between total MFI at a defined peptide concentration and background MFI derived from ligand-free class I expression to the same RMA-S cells) using nonlinear regression analysis fitted to classical Michaelis-Menten kinetics (Prism 3.02; GraphPad Software, San Diego, CA). Nonlinear Michaelis-Menten regression analysis was preferred because Scatchard transformation, which uses linear regression, amplifies any variation of the data from the linear curve.

Results

Combinatorial peptide library screening refines the H4[b] mimotope

Two previous studies defined the mimotopes recognized by H2K[b]-restricted, H4[b] minor H Ag-specific CTL line M9 (25, 29). They revealed that the epitope is eight amino acid residues long and neutral in charge (25, 29). The mimotopes contained an invariant glycine at position (P) 2 and isoleucine at P3 and P6. They also contained glutamic acid, glutamine, glycine, or serine at P1; isoleucine, tyrosine, or valine at P4; arginine or histidine at P7; and isoleucine, leucine or valine at PΩ, the carboxyl-terminal anchor of H2K[b] binding peptides. Position 5, which contains the dominant H2K[b]-binding peptide anchor, was a phenylalanine, a bias introduced during the construction of the combinatorial library (25, 29). Thus, a library of 72 mimotopes can sensitize targets for M9.

The hydrophobicity and the RP chromatography pattern of mimotopes containing glutamine and glycine at P1 are distinct from the naturally processed H4[b] epitope (data not shown). Further, the natural H4[b] epitope is neutral in charge and is predicted to carry an arginine or histidine at P7 (25, 29). Thus, mimotopes containing glutamine or glycine at P1 are less likely to be the H4[b] epitope. Moreover, the negatively charged glutamic acid at P1 could neutralize the positively charged amino acid residue at P7. Thus, a library of 18 mimotopes (see Table I) were synthesized and tested in a target reconstitution assay using M9 and M11, another H2K[b]-restricted H4[b]-specific CTL clone with divergent TCR Vβ usage. H2K[b]-positive but H4[b]-negative RMA cells pulsed with varying

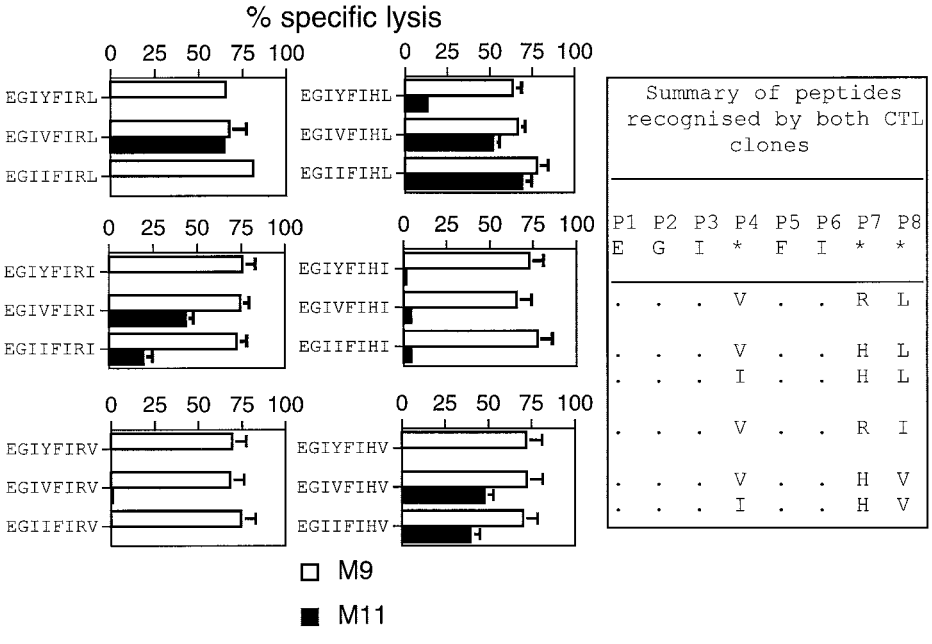

FIGURE 1. The H4[b]-specific CTL epitope predominantly contains a valine at P4 and a histidine at P7. RMA cells were individually pulsed with each of the indicated 18-peptide mimotopes and tested for recognition by 2 H4[b]-specific CTL clones, M9 and M11. Peptides in this library differ by three residues at P4 (isoleucine, valine, or tyrosine), two at P7 (arginine or histidine), and three at PΩ (isoleucine, leucine, or valine). The E:T ratio was 10:1. A summary of the results is presented in the box on the right. Values are representative of two independent experiments, which showed similar results.

concentrations (10^{-14}–10^{-6} M) of individual mimotopes served as target cells in a standard ^{51}Cr-release assay. Both M9 and M11 required a histidine, but rarely arginine at P7 (Fig. 1). Further, peptides with tyrosine at P4 were not recognized by the two H4^b-specific CTL clones (25). Thus, the epitope was narrowed from 18 to four varying only at two positions: EGIVFIHL, EGIIFIHL, EGIVFIHV and EGIIFIHV. However, none of these mimotopes coeluted with the natural H4^b epitope upon RP chromatography (using gradient 2) because they were more hydrophobic (Table I).

Because P2G, P3I, P6I, and P7H are critical for recognition of the H4^b epitope by both CTL clones (25), we reasoned that residues at P1, P4, and P5 could be altered to lower the hydrophobicity of the mimotopes. Thus, a library was generated of peptides containing amino acid residues at P1, P4, and P5 that have the potential to lower the hydrophobicity of the peptide and to neutralize the positive charge of P7His. Target reconstitution assay using both M9 and M11 revealed that replacements with serine at P1, valine at P4, tyrosine at P5, and leucine at PΩ resulted in efficient CTL recognition (Fig. 2*A*, *top*).

To determine whether any of the four mimotopes (Fig. 2*A*, *top*) coelute with the natural H4^b epitope, total cellular peptides were isolated and fractionated by RP chromatography, and pools of eight fractions were tested in a target reconstitution assay using M9 and M11 as probes. Two peptide pools sensitized targets for M9 and M11 recognition (Fig. 2*B*, *first panel*). Resolution of the fractions constituting the active pools revealed that fractions 44–46 contain M9 and M11 epitopes (Fig. 2*B*, *second panel*). Fractions 64 and 65 contained an additional M11 epitope(s), which was not detected by M9. The second activity was observed when the peptides were fractionated using a mixture of C18 and cation exchange chromatography matrices (Fig. 2*B*), but not when resolved with C18 matrix alone (see Figs. 3, 5, and 7). In the same experiment, 100 fmol of the four M9 and M11 active synthetic peptides were also fractionated by RP chromatography. The resulting fractions adjacent to the natural epitope (Fig. 2*B*, *second panel*) were tested in target reconstitution assay. One of the four mimotopes tested, SGIVYIHL, eluted close to the natural epitope but was more hydrophobic (Fig. 2*B*, *fourth panel*).

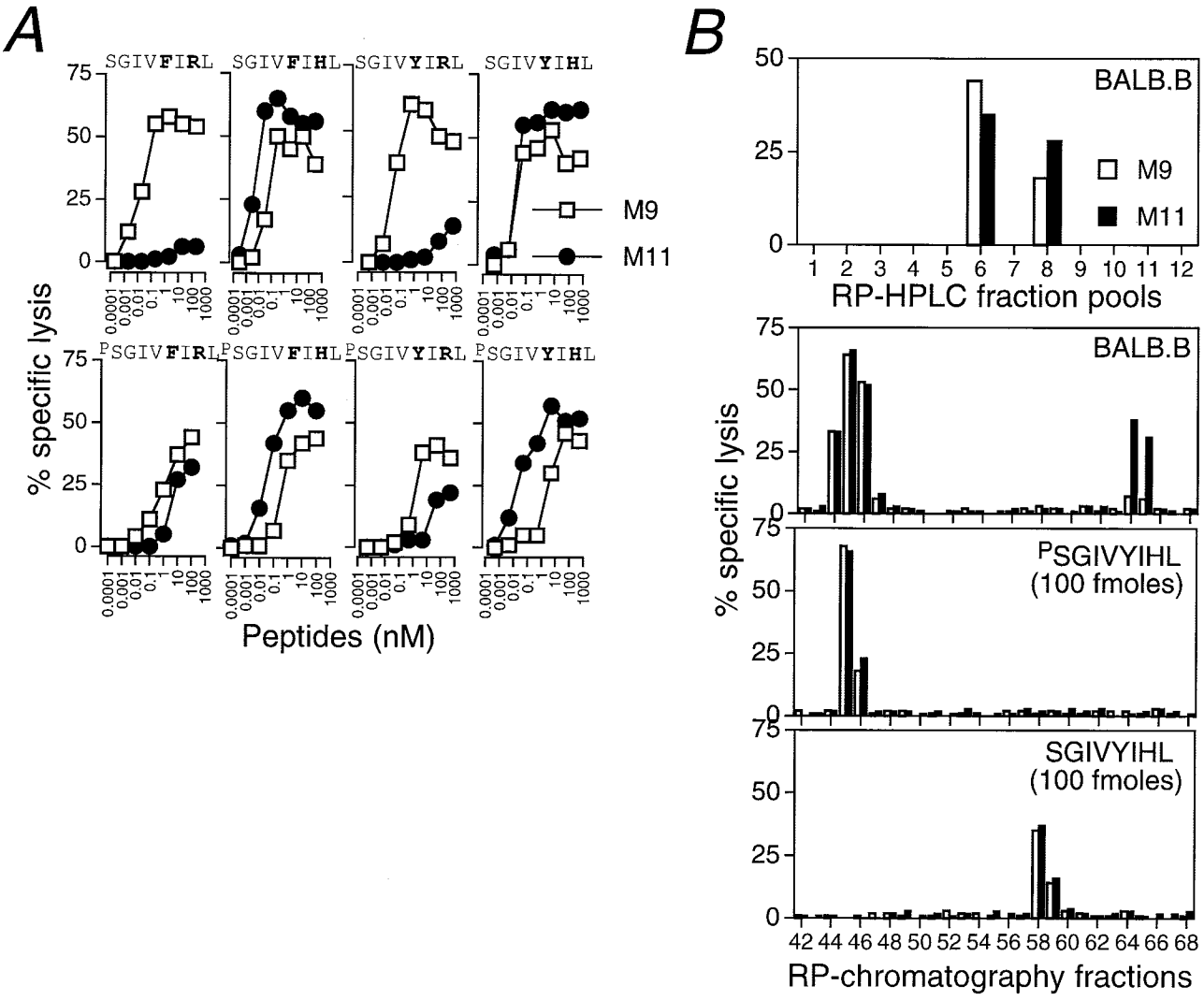

FIGURE 2. The naturally processed H4^b-specific CTL epitope may be a phosphopeptide. *A*, Ten-fold serial dilutions between 1 μM and 0.1 pM of each peptide mimotope were incubated with ^{51}Cr-labeled RMA cells and tested in a ^{51}Cr release assay. The concentration of the synthetic peptide resulting in half-maximal lysis is represented in Table II. *B*, Total cellular peptides were extracted from 15 BALB.B spleens and fractionated by RP chromatography through a mixture of C18 and cation exchange matrices using gradient 1. Pools of eight fractions were first tested in a target reconstitution assay using M9 (□) and M11 (■) CTL clones to determine the fraction(s) containing the H4^b epitope (*first panel*). Fractions 42–68, constituting the active pool and its flanks were further tested in target reconstitution assay as described above (*second panel*). At the same time, synthetic ^{P}SGIVYIHL (*third panel*) and SGIVYIHL (*fourth panel*) were also fractionated and tested as above. E:T = 10; representative of two similar experiments.

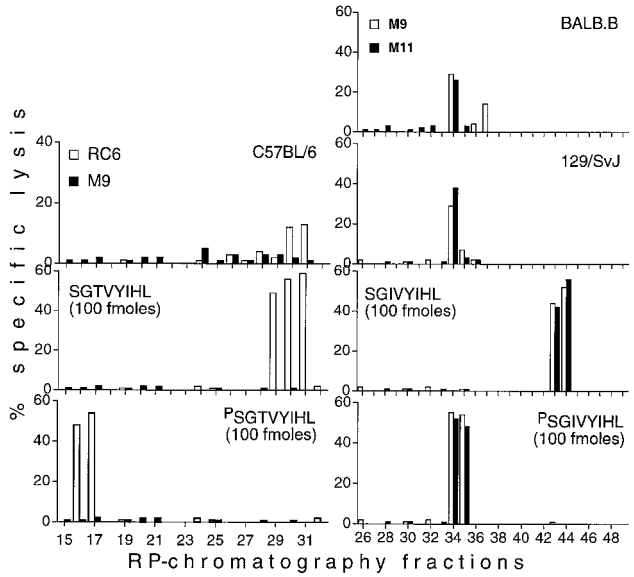

FIGURE 3. P3Ile to Thr variation in the H4 epitope causes minor H Ag-specific alloreactivity. Total cellular peptides were extracted from five C57BL/6, five BALB.B, and five 129 spleens and fractionated by RP chromatography through a C18 column using gradient 1 (see *Materials and Methods*). For comparison, 100 fmol of synthetic ^{P}SGTVYIHL (^{P}H4^{a}; *left bottom panel*), ^{P}SGIVYIHL (^{P}H4^{b}; *right bottom panel*), SGTVYIHL (H4^{a}; *left middle panel*) and SGIVYIHL (H4^{b}; *right third from top panel*) were also resolved. Fractions previously determined to contain the natural and synthetic peptides were tested as in Fig. 2B. E:T = 10; representative of two similar experiments.

Phosphorylation of P1Ser or P5Tyr could lower the hydrophobicity of the mimotope and also neutralize the positive charge of P7His. Therefore, phosphorylated peptides were tested in the target reconstitution assay using both M9 and M11. All of the phosphorylated peptides were recognized by the two H4^{b}-specific CTL clones albeit with varying sensitivity (Fig. 2*A, bottom*). One of the four phosphorylated peptide, ^{P}SGIVYIHL (P1^{P}Ser), migrated coincident with the natural H4^{b} epitope on RP chromatography (Fig. 2*B, third panel*). Contrary to expectations, SGIV^{P}YIHL (P5^{P}Tyr) peptide was more hydrophilic than the P1^{P}Ser peptide; hence, the P5^{P}Tyr peptide eluted earlier than the latter (Table II). These data suggest that ^{P}SGIVYIHL may be the natural H4^{b} epitope.

Emp-3 *encodes allelic H4 epitopes*

To identify the gene that encodes the H4^{b} epitope, a tblastn search for SGIVYIHL was conducted within the translated nucleotide sequence bank (http://www.ncbi.nml.nih.gov). This search revealed cent-per-cent (100%) identity with aa 152–159 of a 163-residue-long Emp-3 derived from 129 strain (Table III and Ref. 30). The *Emp-3* gene maps to the region of mouse chromosome 7 predicted for *H4^{b}* (31). To determine the basis of H4 disparity between C57BL/6 responder and BALB.B and 129 stimulators, a 166-bp segment of *Emp-3*, which encodes the region surrounding the H4^{b} epitope, was amplified by PCR from genomic DNA. Nucleotide sequence analysis revealed an intact 166-bp nucleotide sequence but for a single T (ATT in BALB.B, similar to that reported for 129) to C (ACT in C57BL/6) polymorphism within codon 154 of *Emp-3* (Table III). This single nucleotide polymorphism results in an isoleucine to threonine change at P3 (the auxiliary anchor for H2K^{b}-binding peptides) of the H4 epitope (Table III).

Previous studies assumed that C57BL strains carry a *H4* null allele and hence do not elicit CTL responses (32). However, immunization of B10.129-*H4^{b}* mice with splenocytes from the intra-H4 region recombinant strain B10.129-RC6 (33) resulted in a weak CTL response from which a CTL line RC6 was derived.

Table II. *List of peptides used in this study and their physical and biological properties:* second-generation mimotopes[a]

Sequence	Hydrophobic Index[a]	RP Chromatography Retention Time (Gradient[b])	Peptide Concentration at Half-Maximum Lysis ($\times 10^{-12}$ M)[c]	
			M9	M11
Natural H4^{b}	<0.95[d]	47.7		
TGIVFIRL	0.94	ND[f]	400/700	4,000/7,000
TGIVFIHL	1.09	ND	500/200	10/20
^{P}TGIVFIRL	<0.94	ND	2,000/3,000	40,000/30,000
^{P}TGIVFIHL	<1.09	ND	1,000/2,000	30/200
TGIVYIRL	0.84	ND	100/100	1,000/1,000
TGIVYIHL	0.98	ND	300/200	30/20
^{P}TGIVYIRL	<0.84	ND	2,000/2,000	90,000/100,000
^{P}TGIVYIHL	<0.98	ND	3,000/4,000	300/400
SGIVFIRL	0.91	ND	20/30	20,000/20,000
SGIVFIHL	1.05	58.5	70/30	7/2
^{P}SGIVFIRL	<0.91	ND	1,000/1,000	3,000/5,000
^{P}SGIVFIHL	<1.05	57.3	350/2,000	35/20
SGIVYIRL	0.80	ND	30/10	3,000/3,000
SGIVYIHL	0.95	56.2	20/20	20/40
^{P}SGIVYIRL	<0.80	ND	1,000/2,000	10,000/20,000
^{P}SGIVYIHL	<0.95	47.7	1,000/3,000	20/200
SGIV^{P}YIHL	<0.95	45.0	700	700

[a] Based on Ref. 47.

[b] RP chromatography conditions used to separate peptides in Tables I and II were different; the columns used for chromatography of peptides described in this table and Fig. 2 are different from that used for peptides fractionated in Figs. 3, 5, and 7. See *Materials and Methods* for details. Also, the two numbers in this column correspond to the elution times obtained from two similar RP chromatographies of the same mimotope.

[c] Determined as described in Fig. 2 with both H4^{b}-specific M9 and M11 CTL clones. The two values reflect two independent determinations.

[d] Based on the hydrophobicity of the mimotope that migrates closest to the natural epitope.

[e] Based on Ref. 29.

[f] ND, not determined because the hydrophobic index of these peptides was higher than the natural Ag and/or the biological activity was elicited by very high amounts of the mimotopes.

Table III. *H4 alleles and homologues*

Species/ Strain	Sequence[a]								Ref.
Codons	152	153	**154**	155	156	157	158	159	
C57BL/6	agc	ggc	**act**	gtc	tac	atc	cac	ctg	
	Ser	Gly	**Thr**	Val	Tyr	Ile	His	Leu	This study
BALB.B	agc	ggc	**att**	gtc	tac	atc	cac	ctg	
	Ser	Gly	**Ile**	Val	Tyr	Ile	His	Leu	This study
129	agc	ggc	**att**	gtc	tac	atc	cac	ctg	
	Ser	Gly	**Ile**	Val	Tyr	Ile	His	Leu	30
Rat	ag***t***[b]	ggc	atc	atc	tac	atc	cac	ctg	
	Ser	Gly	**Ile**	**Ile**	Tyr	Ile	His	Leu	30
Human	agc	ggc	atc	atc	tac	atc	cac	ct***a***[b]	
	Ser	Gly	**Ile**	**Ile**	Tyr	Ile	His	Leu	30

[a] Codons are depicted above the three-letter amino acid code.
[b] Silent mutation.

Using RC6, the natural $H4^a$ epitope was identified. Thus, peptides were extracted from C57BL/6, BALB.B, or 129 splenocytes as described in Fig. 2. After RP chromatography, the fractions containing $H4^a$- and $H4^b$-specific CTL epitopes were identified by testing individual fractions in the CTL target reconstitution assay. Simultaneously, 100 fmol of synthetic nonphosphorylated and phosphorylated peptides were fractionated and tested. The data revealed that the two epitopes had distinct RP chromatography patterns (Fig. 3). Further, the data revealed that the $H4^a$-derived CTL epitope appears nonphosphorylated SGTVYIHL and confirmed that the $H4^b$-derived CTL epitope is ^{P}SGIVYIHL ($^PH4^b$; Fig. 3). Thus, the allelic H4 minor H peptides differ at least by a hydroxyl to methyl change at P3.

To affirm that the $H4^a$ and $H4^b$ epitopes are derived from Emp-3, a minigene encompassing exon 5 of this gene was isolated from C57BL/6 and BALB.B genomic DNA and cloned into a mammalian expression vector. Transfer of the minigene constructs into the H4-negative B6/wt19 line (Fig. 4, *left*) conferred it sensitive to $H4^a$- (Fig. 4, *middle*) or $H4^b$-specific CTL clones (Fig. 4, *right*). Thus, *Emp-3* does indeed encode the H4 minor H Ag.

$H4^b$ but not the $H4^a$ epitope is a phosphopeptide

To confirm that the naturally processed $H4^b$ epitope is a phosphopeptide, one half of the acid extract from C57BL/6 and 129 splenocytes was treated with a mixture of phosphatase inhibitors, and the other half was treated with alkaline phosphatase. After 1 h, the reactions were stopped, and peptides were isolated and fractionated. Fractions containing CTL epitopes were identified as described above. Alkaline phosphatase did not alter the elution time of the $H4^a$ epitope on RP chromatography (Fig. 5, *top two panels*). However, in the same experiment, alkaline phosphatase altered the migratory pattern of $H4^b$ epitope (Fig. 5, *bottom two panels*). Dephosphorylated $H4^b$ eluted at the same time as the synthetic nonphosphorylated $H4^b$ peptide (data not shown; but compare bottom two panels in Fig. 5 with the third right panel in Fig. 3). Thus, the naturally processed $H4^b$ epitope but not the $H4^a$ peptide is phosphorylated.

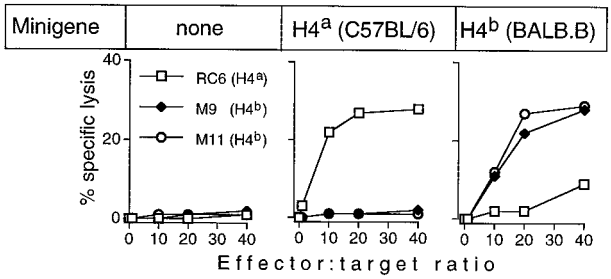

FIGURE 4. Minigene complementation of H4-negative cell line confers it sensitive to $H4^a$- and $H4^b$-specific CTL clones. A 166-bp cDNA corresponding to exon 5 of *Emp-3* was isolated and used to construct an H4 minigene. An authentic minigene cDNA, affirmed by nucleotide sequence analysis, encoding $H4^a$ and $H4^b$, was individually transfected into B6/wt19, an SV40-transformed, $H2^b$-positive, but H4-negative fibroblast line (*left*). A day later, the transfectants were used as targets for $H4^a$ (RC6)- and $H4^b$ (M9 and M11)-specific CTL clones. BALB.B- and C57BL/6-derived minigene-transfected cells served as the negative controls for RC6 and M9 and M11, respectively.

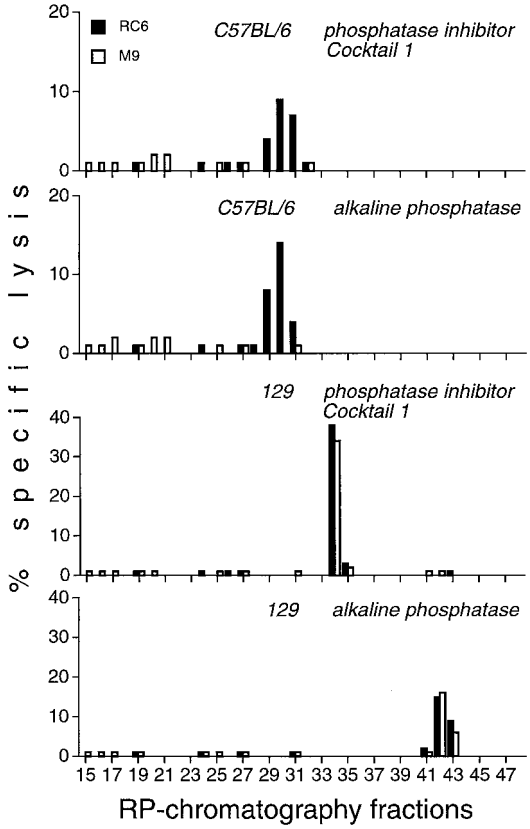

FIGURE 5. The natural $H4^b$ but not the $H4^a$ epitope is phosphorylated because it is sensitive to alkaline phosphatase. Acid extracts of five C57BL/6 (*top two panels*) and five 129 (*bottom two panels*) splenocytes were divided in two equal aliquots. One aliquot was treated with the phosphatase inhibitor cocktail 1 (*first* and *third panels*) or with alkaline phosphatase (*second* and *fourth panels*). Enzyme activity was stopped; peptides were isolated and fractionated. The fractions containing $H4^a$ and $H4^b$ epitopes were identified as in Fig. 2*B*. Natural $H4^a$, $^PH4^b$, and dephosphorylated $H4^b$ peptides elute at times similar to that expected for synthetic $H4^a$, $^PH4^b$, and $H4^b$, respectively (see Fig. 3).

A P3Thr to isoleucine change explains the $H4^b$ minor H Ag

To determine whether the P3Thr to isoleucine change in the SGTVYIHL peptide is sufficient to explain the $H4^b$ alloantigen, we measured the half-maximal concentration of both phosphorylated and nonphosphorylated $H4^b$ and $H4^a$ peptides required to sensitize $T2K^b$ targets for lysis by bulk CTL from C57BL/6 mice immunized with BALB.B minor H Ags. Only the $H4^b$-reactive CTL showed robust cytolytic activity against both $H4^b$ and $^PH4^b$ peptides (Fig. 6, *top*). As commonly observed with allelic minor H Ags, lysis against the $H4^a$ peptides was also observed but at several orders of magnitude lower peptide concentration (Fig. 6, *bottom*). These results suggested that the H4 alloantigen is primarily explained by the genetically determined P3Thr to isoleucine change

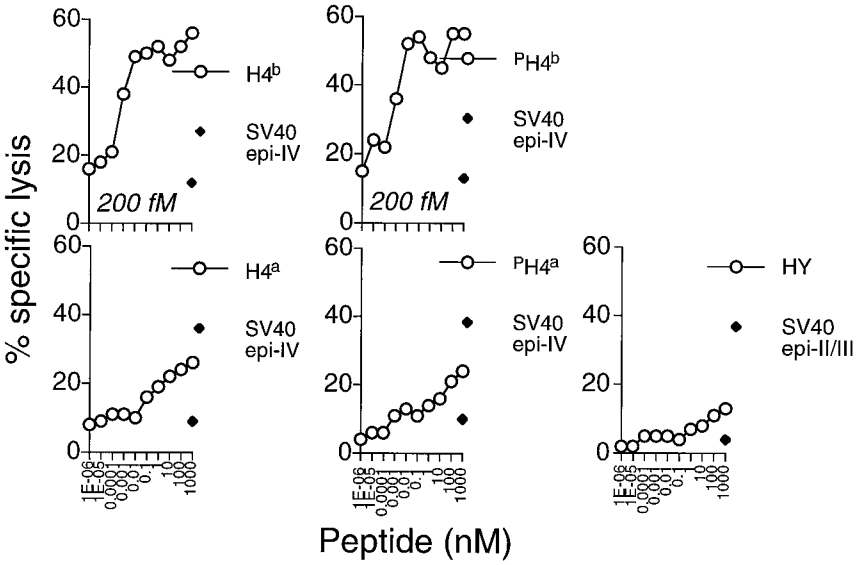

FIGURE 6. Sensitive recognition of $H4^b$ by BALB.B-immune splenocytes. Female C57BL/6 mice were immunized twice at 7-day intervals with BALB.B male splenocytes. Seven days after immunization, splenocytes from responder mice were stimulated in vitro with irradiated BALB.B spleen cells for 5 days in a mixed leukocyte culture. Effector cells from the mixed leukocyte culture were analyzed for specific cytotoxicity against the indicated peptide-loaded T2 target cells. Peptide concentration eliciting half-maximal lysis is indicated within each panel where saturation was achieved. E:T = 50. SV40 epi-IV, $H2K^b$-restricited VVYDFLKL; SV40 epi-II/III, $H2D^b$-restricted CKGVNKEYL; ◆, 1.0 μg of SV40 epitope controls, which elicited background levels of killing.

in the octameric H4 peptide, with no obvious contribution by phosphorylation of P1Ser.

Conservation of $H4^b$ extends to humans

Comparison of mouse *Emp-3* with the rat and human homologues revealed an isoleucine substitution for P4Val (Table III) (30). To determine whether human cells present $H4^b$ Ag to specific CTL, first, Jurkat-K^b (Jurkat cells expressing $H2K^b$) were tested in a cytolytic assay using M9 CTL. The $H4^b$-reactive M9 specifically recognized Jurkat-K^b (Fig. 7*A*). To confirm that a specific peptide that is more hydrophobic than the mouse $H4^b$ was being presented by Jurkat-K^b, peptides from the human cell line were acid extracted. One half of the extract was treated with a phosphatase inhibitor mixture, and the other half was treated with alkaline phosphatase. Peptides were isolated after stopping the reaction and fractionated, and the human H4 epitope was identified as above. Synthetic phospho- and nonphosphopeptides corresponding to human H4 were also resolved by RP chromatography and CTL active fractions were identified. Two sets of $H4^b$-specific CTL activities were isolated from Jurkat-K^b peptides treated with the phosphatase inhibitor mixture (Fig. 7*B, top two panels*). The two activities corresponded with the synthetic human H4 phospho- and nonphosphopeptides, respectively (Fig. 7*B, bottom two panels*). On alkaline phosphatase treatment, the majority of the natural human H4 phosphopeptide eluted coincident with the nonphosphorylated synthetic peptide (Fig. 7*B, second from top panel* and *bottom panel*). Thus, the naturally processed human H4 epitope is a phosphopeptide akin to the naturally processed BALB.B- and 129-derived $H4^b$ epitope. Moreover, the expression of $H4^b$ by a human cell line is consistent with the transspecies evolution of this alloantigen (30, 34).

$H4^b$ is immunodominant

Previous studies indicated that in the C57BL/6 anti-BALB.B response, H60-reactive CTL dominates over other minor H Ags (8, 15–17, 35). However, at that time the magnitude of the $H4^b$ response was difficult to ascertain accurately because the epitope was hitherto unknown. To determine the number of $H4^b$-reactive CTL, $H2K^b$-$H4^b$, $H2K^b$-$^PH4^b$, $H2K^b$-H60, and $H2D^b$-HY tetramers were generated. All tetramers reacted specifically with their respective CTL clones (15). To generate a CTL response to multiple minor H Ags, C57BL/6 mice were immunized twice at 7- to 10-day intervals with BALB.B splenocytes. Seven days after the booster immunization, ex vivo splenocytes were stained with tetramer-PE along with anti-CD8a-FITC and analyzed by flow cytometry. Both $H2K^b$-$^PH4^b$ and $H2K^b$-H60 tetramers specifically bind the elicited $CD8^+$ T cells (Fig. 8*A, first* and *third panels*). In contrast, $H2K^b$-$H4^b$, the tetramer made using nonphosporylated $H4^b$ peptide, and $H2D^b$-HY tetramer-reactive $CD8^+$ T cells were scarcely found (Fig. 8*A, second* and *fourth panels*).

To determine whether in vitro Ag stimulation caused the BALB.B-immune splenocytes to recognize $^PH4^b$ as well as $H4^b$ peptides in the cytolytic assay, tetamer binding was performed on the same splenocytes after stimulation with Ag (H60 or $H4^b$ congenic splenocytes) in a 5-day mixed leukocyte culture. The data revealed that both $H2K^b$-$^PH4^b$ and $H2K^b$-$H4^b$ tetramers react with polyclonal CTL generated after stimulation of BALB.B-immune splenocytes in in vitro mixed leukocyte culture (Fig. 8*B*). Additionally, $H2K^b$-$H4^b$ tetramer reacts with ex vivo BALB.B-immune splenocytes at 4°C (data not shown) but loses reactivity at 37°C (Fig. 8*A*), suggesting low affinity interaction of the nonphosphorylated H4 alloantigen with its receptor. Thus, the anti-$H4^b$ CTL response is directed against the phosphorylated $H4^b$ epitope in vivo.

To understand the position of $H4^b$ in the immune dominance hierarchy, the physicochemical properties of $H4^b$, $^PH4^b$, and HY epitopes were analyzed and compared with H60. First, the copy number of $H4^b$ epitope was compared with that of H60. To accomplish this, the fraction(s) containing the H60 CTL epitope was identified (data not shown). Fractions containing $H4^b$ and H60 epitopes were pooled, and 2-fold dilutions were tested in a target cell reconstitution assay. Titration of BALB.B and 129 splenocytes suggested that they expressed similar numbers of $H4^b$ epitope, which was comparable with results for H60 (Fig. 9*A*).

The affinity between the MHC class I molecule and the epitope as well as that of the TCR and its cognate Ag (p/MHC) are critical parameters required to estimate the copy number of peptide epitopes presented by a cell. To establish the relative affinities of the peptide epitopes for class I molecules, peptide concentrations required to stabilize them on the surface of TAP-deficient RMA-S cells was determined. From the binding isotherms, the relative affinity (K_d) of peptide class I interaction was calculated (see *Materials and Methods*). Both the $H4^b$ and $^PH4^b$ epitopes demonstrated a higher binding affinity for $H2K^b$ than did $H4^a$ (Fig. 9*B, left*). The phosphopeptide bound with a stronger affinity than did the nonphosphorylated version (Fig. 9*B*). Moreover, $H4^a$ bound to

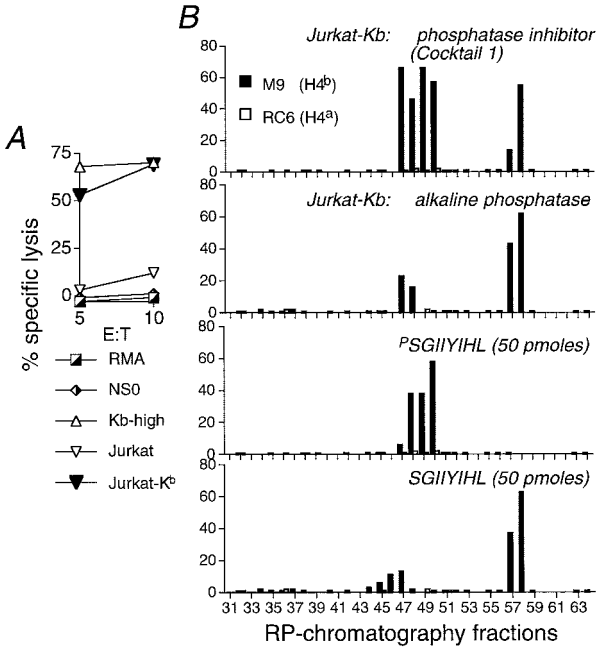

FIGURE 7. Human cells also express $H4^b$, which is phosphorylated and has an altered mobility by RP chromatography owing to a P4Val to Ile variation. *A*, The expression of $H2K^b$-restricted $H4^b$ epitope was tested using ^{51}Cr-labeled cells expressing the class I molecule in the appropriate background using $H4^b$-specific CTL clone M9 as the probe. RMA is a C57BL/6 ($H2^b$) thymoma, NS0 is a BALB/c ($H2^d$) plasmacytoma, and Jurkat is a human $CD4^+$ T cell line. Kb-high and Jurkat-K^b were derived by transfection of the $H2K^b$ cDNA into the NS0 and Jurkat cells, respectively. E:T ratio in the assay is indicated. *B*, Acid extracts of Jurkat-K^b cells were treated with phosphatase inhibitors or alkaline phosphatase as in Fig. 5. After the reaction was stopped, peptides were isolated and fractionated along with synthetic phosphorylated and nonphosphorylated peptides. Human H4 epitope was identified as in Fig. 2*B*. These experiments were performed twice with identical results.

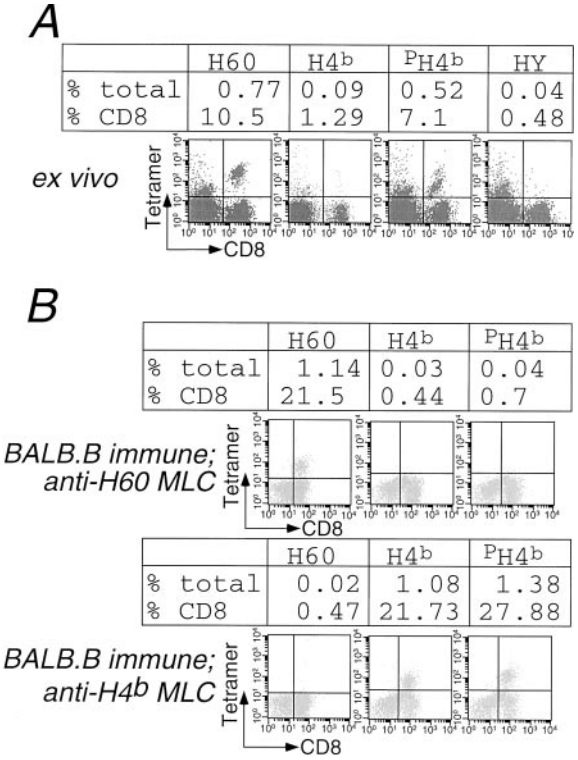

FIGURE 8. Enumeration of minor H Ag-reactive $CD8^+$ T lymphocytes. *A*, After immunization of female C57BL/6 mice with BALB.B male spleen cells, splenocytes were stained ex vivo with $H2K^b$-H60, $H2K^b$-$H4^b$, $H2K^b$-$^PH4^b$, and $H2D^b$-HY tetramer-PE along with anti-CD8a-FITC and analyzed by flow cytometry. *B*, BALB.B-immune C57BL/6 splenocytes were stimulated either with H60 (*top panels*) or $H4^b$ (*bottom panels*) congenic spleen cells in vitro for 5 days. $H2K^b$-H60, $H2K^b$-$H4^b$, and $H2K^b$-$^PH4^b$ tetramer-reactive $CD8^+$ T lymphocytes were analyzed as above. The percentages of tetramer-positive cells are indicated above the dot plots. Data are representative of at least two experiments.

$H2K^b$ more efficiently than the HY and H13 epitopes bound to $H2D^b$ (Fig. 9*B*). These results suggested the following affinity hierarchy for class I peptide binding: $H60 \approx {}^PH4^b > H4^b > HY$.

The TCR of $H4^b$- and H60-specific CTL clones used show similar relative avidities (R. Yadav, Y. Yoshimura, G. J. Christianson, W. V. Ajayi, R. Shashidharamurthy, D. C. Roopenian, and S. Joyce, manuscript in preparation). Previous estimates suggested that ~15 copies of the H60 epitope are presented per BALB.B splenocyte (7). Considering that $^PH4^b$ and H60 bind $H2K^b$ with similar affinities and that the Ag (p/MHC) binds their cognate TCR with similar avidities, our results suggest that $H4^b$ and H60 epitopes are expressed in similar copy numbers by BALB.B and 129 splenocytes (Fig. 9*A*). Taken together the data suggest that $H4^b$ is approximately as dominant as H60.

Discussion

Until now, combinatorial peptide library screening has successfully led to mimotopes with some similarity to MHC-bound natural ligands, but never to the precise peptide epitope (25, 29, 36–39). We show here that this approach, combined with careful attention to the biochemical properties of the naturally processed epitope, resulted in the direct identification of the long sought after immune dominant $H2K^b$-restricted $H4^b$ epitope (1, 2, 29). Like most other identified autosomally encoded minor H Ags, H4 arises from a single amino acid (P3Thr to isoleucine) change in a conserved, broadly expressed protein (35). This results in a processed peptide that binds $H2K^b$ avidly. Unlike any known naturally processed self peptide, this allelic change results in the differential phosphorylation of the peptide epitope. Alloantigenicity of germline-encoded H4 peptides is exaggerated by posttranslational modifications.

Much of the alloantigenicity of the H4 allelic variants can be accounted solely by the P3Thr to isoleucine change in the H4 [SG(T/I)VYIHL] epitope. Most changes resulting in incompatibility are in amino acids that directly interact with critical TCR contact residues (10, 11). The results presented herein provide functional evidence for the role of secondary anchor residues, such as those at P3, in altering the antigenicity of the class I-peptide complex. Although this change altered the binding affinity of the H4 analogs to $H2K^b$, the extent to which this lowered binding affinity impacts the resulting T cell response is currently unknown. A thorough x-ray crystallographic analysis of HLA-A2 individually complexed with five different peptides revealed that secondary anchors influence the orientation of solvent exposed residues without altering the conformation of the dominant anchors or inducing any significant variation in the HLA-A2 structure (40). The orientation of the solvent exposed peptide residues albeit altered were accessible to the TCR, and it was concluded that interactions with such residues would determine the antigenicity of the class I-peptide complex (40). A similar finding was reported independently in an MHC class II self Ag model (41). An allelic MHC class II-restricted Ag, mouse hemoglobin elicits an alloreactive $CD4^+$ T cell response. This reactivity is due to E73D change, i.e., a loss of a methylene group in a residue that contacts pocket 6 of $H2IE^k$. The effect of this change was transmitted up to 10 Å distal to the altered

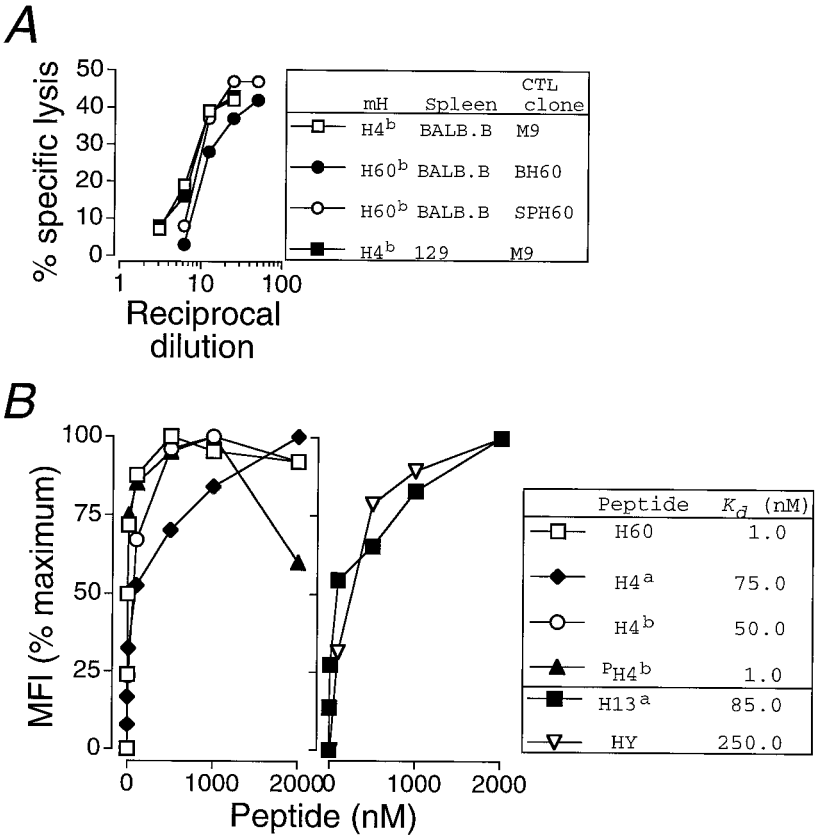

FIGURE 9. High affinity interaction between $H2K^b$ and $^PH4^b$. *A*, The fractions that contained $^PH4^b$ (see Fig. 3) and H60 (data not shown) epitopes were pooled, and serial 2-fold dilutions were tested for CTL target reconstitution activity. The H60 epitope is more hydrophobic and migrates adjacent to the $H4^b$ epitope when extracted from BALB.B or 129 splenocytes (data not shown). *B*, Before pulsing RMA-S cells with peptides, they were maintained at 26°C for 18–20 h. Increasing concentrations of indicated peptides were incubated with RMA-S cells for 45 min at 26°C, washed, and incubated at 37°C for an additional 4 h. SV-40 epi-IV in the case of $H2K^b$ or SV-40 epi-II/III in the case of $H2D^b$ and $H2K^d$-restricted influenza peptide were used as positive and negative controls, respectively. After removal of dissociated peptides, $H2K^b$ and $H2D^b$ were stained with biotinylated mAb specific for the class I molecule. Class I expression was monitored by flow cytometry. From the resulting binding isotherms, the relative affinity (K_d) of peptide class I interaction was calculated (see *Materials and Methods*).

residue 73 (41). Similarly, the isoleucine to threonine change at P3 could influence TCR recognition indirectly and hence confer allelic discrimination by anti-H4 T cells.

When acid extracts of peptides were resolved using a mixture of C18 and cation exchange matrices, a few late eluting fractions activate M11 but not M9 CTL clones. This activity is lost when peptides are fractionated with C18 matrix alone, suggesting the recognition of a more cationic (positive charged) ligand. The second M11 ligand is distinct from that observed in Jurkat-K^b in that the latter elutes coincident with the nonphosphorylated human H4 peptide. The second M11-specific ligand could be a precursor of the natural $^PH4^b$ epitope or a distinct peptide unrelated to the Emp-3-derived epitope. If a precursor, it is not the nonphosphorylated $H4^b$ because the second ligand elutes after the nonphosphorylated $H4^b$ peptide. Therefore, we predict that a longer peptide precursor imparts the second M11-specific activity. Interestingly, C terminal to the $H4^b$ epitope is a stretch of three basic arginine-lysine-arginine residues in the Emp-3 sequence (30). Peptides longer than the processed epitopes are known to bind MHC class I molecules (42, 43) and be recognized by CTL (44). Therefore, it is conceivable that M11 recognizes a longer peptide precursor in addition to the processed $^PH4^b$ epitope. M11 could recognize this precursor either directly or after further processing on addition to cells in the reconstitution assay.

The degree to which natural phosphorylation could influence immunogenicity of peptide epitopes is of considerable importance. Anderson et al. (45) have shown that TAP transports phosphopeptides, binds class I molecules, and elicits CTL responses. Further, Zarling et al. (46) demonstrated that phosphopeptides, predominantly phosphoserine-, phosphothreonine-, and, less frequently, phosphotyrosine-containing peptides, assemble with human MHC class I molecules and elicit CTL response in vitro. Curiously, BALB.B-immune splenocytes did not discriminate between $H4^b$ and $^PH4^b$ in cytolytic assays. However, $H2K^b$ tetramers containing $^PH4^b$ but not $H4^b$ bind the elicited $CD8^+$ T cells. A plausible explanation for this disparity is that tetramer binding was performed on freshly isolated BALB.B-immune splenocytes, whereas CTL responses were determined in the same splenocytes after in vitro stimulation with Ag in a 5-day mixed leukocyte culture. In vitro stimulation and expansion of CTL in culture may have resulted in broadening of $H4^b$-specific CTL reactivity, which now includes nonphosphorylated $H4^b$ recognition. In support of this explanation is the fact that $H2K^b$-$H4^b$ and $H2K^b$-$^PH4^b$ tetramers do indeed react with polyclonal CTL generated after in vitro stimulation of BALB.B-immune splenocytes in a mixed leukocyte culture. Additionally, the binding of $H2K^b$-$H4^b$ tetramer to ex vivo BALB.B-immune splenocytes appears to be of low affinity. Taken together, the data suggest that the anti-$H4^b$ CTL response is directed against the phosphorylated $H4^b$ epitope in vivo. Further, an increased efficiency of TCR engagement conferred by the P1Ser phosphorylation of $^PH4^b$, which is absent from the natural $H4^a$ analog, could also increase the foreignness of the $H4^b$ Ag and possibly contribute to its immune dominance.

In conclusion, our results support the notion that class I-restricted phosphopeptides operate as epitopes in vivo. Because differential protein phosphorylation underlies various biological and pathological processes including tumorigenesis, phosphopeptides could be harnessed in tumor-specific vaccine design. Thus, a detailed understanding of differential presentation of naturally processed phosphopeptides by MHC class I molecules under physiological and pathophysiological states is warranted because of its therapeutic value.

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