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The Majority of Immunogenic Epitopes Generate CD4$^+$ T Cells That Are Dependent on MHC Class II-Bound Peptide-Flanking Residues[1]

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Peptides bind to MHC class II molecules with a defined periodicity such that the peptide-flanking residues (PFRs) P-1 and P11, which lie outside the core binding sequence (P1–P9), are solvent exposed and accessible to the TCR. Using a novel MHC class II:peptide binding assay, we defined the binding register for nine immunogenic epitopes to formally identify the flanking residues. Seven of the nine epitopes, restricted by H-2A^k, H-2A^{g7}, or H-2E^k, were found to generate T cells that were completely dependent on either P-1 or P11, with dependency on P-1 favored over P11. Such PFR dependency appears to be influenced by the type of amino acid exposed, in that residues that can form salt bridges or hydrogen bonds are favored over small or hydrophobic residues. Peptides containing alanine substitutions at P-1 or P11 in place of PFRs that mediate dependency were considerably less immunogenic and mediated a substantially reduced in vitro recall response to the native protein, inferring that PFR recognition increases immunogenicity. Our data suggest that PFR recognition is a common event characteristic of all MHC class II-restricted T cell responses. This key feature, which is not shared by MHC class I-restricted responses, may underlie the broad functional diversity displayed by MHC class II-restricted T cells. ***The Journal of Immunology,*** **2002, 169: 739–749.**

The idea that peptides bind to MHC class II molecules with a common pattern was first proposed by Rothbard and Taylor in 1988 (1). There is now general agreement from structural studies that MHC class II-bound peptides adopt an extended type II polyproline conformation with a common periodicity, such that residues at P1, P4, P6, and P9 point down into MHC pockets, whereas P-1, P2, P5, P8, and P11 are solvent exposed and point toward the TCR (2–13). This is in part due to the extensive, and often conserved, interaction with the peptide backbone. Naturally processed MHC class II-bound peptides possess ragged amino (NH_2) and carboxy (COOH) termini (14–18). These amino acids have been referred to as peptide-flanking residues (PFRs)[3] (19, 20) because they are outside the MHC anchor residues (P1, P4, P6, and P9) but within the MHC binding groove (e.g., P-2, P-1, P10, P11). Interestingly, PFRs are a unique and exclusive feature of peptides bound to MHC class II molecules, in that MHC class I-bound peptides are predominantly of fixed length and do not contain PFR extensions.

Although side chain substitutions in PFRs rarely affect peptide binding affinity, the consequence of removing an entire residue is variable. For instance, structural studies have shown that MHC: peptide backbone interactions also involve PFRs (4, 5, 7) and that P-1 in particular appears to be important for peptide stability (21). In contrast, residues at P10 and P11 are frequently removed by Ag processing (7, 17, 22). It has also been noted that the identity of the PFR can contribute to the efficiency of Ag processing (20, 23).

Until recently, it was not clear whether PFRs could be recognized by the TCR, modulate T cell responses, or influence immunogenicity directly. We have shown that a significant percentage of hen egg lysozyme (HEL) 48–63-specific T cells are dependent on the P11 COOH-terminal PFR W62, which results in a profound restriction of TCR-Vβ usage (19, 24). What remains unclear is whether PFR recognition is an infrequent occurrence or a common event characteristic of TCR recognition of MHC class II:peptide complexes. Invariably, the PFRs at P-1 and P11 of all of the MHC class II:peptide complexes solved to date are solvent exposed and accessible to the TCR. Thus, we would hypothesize that most T cell epitopes have the capacity to generate PFR-dependent T cells and that PFR recognition may be restricted to P-1 and P11.

Several factors have been shown to influence immunogenicity and immunodominance, including Ag processing, MHC binding, and the TCR repertoire (20, 25–28). Our initial data suggested that PFRs may be another contributing factor because the substitution or removal of the HEL 48–63 W62 PFR was found to significantly reduce the immunogenicity of this epitope (19). We have also shown that PFR-dependent T cells can be potently antagonized by peptides that lack COOH-terminal PFRs and that these cells respond poorly to native protein or the immunogenic epitope delivered by a recombinant influenza virus (29). These data suggest that Ag processing can generate both stimulatory and antagonist peptides from a single immunogenic epitope. Interestingly, recent studies have shown that background genes in the nonobese diabetic (NOD) mouse, a model for type 1 diabetes, strongly influence

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[3] Abbreviations used in this paper: PFR, peptide-flanking residue; HEL, hen egg lysozyme; NOD, nonobese diabetic; GAD, glutamate decarboxylase; ML, murine lysozyme.

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Ag processing (30). Taken together, these observations suggest that the biology of PFRs may have considerable significance for immunogenicity, T cell immunoregulation, and autoimmunity.

In this study, we defined the peptide binding resister for nine epitopes in HEL and glutamate decarboxylase (GAD) using a novel approach. This analysis allowed us to formally define the flanking residues. We then analyzed T cell responses to these epitopes to address the following questions: 1) what proportion of MHC class II-restricted epitopes generate PFR-dependent T cells; 2) which PFRs are recognized; and 3) does PFR recognition always influence immunogenicity and could it affect immunodominance?

Materials and Methods

H-2A/E:γ2aFc fusion proteins

The basic design of the MHC class II:Ig fusion proteins used in the peptide binding assay is similar to that described by Wucherpfennig and colleagues (31), with modifications. Details of their construction, production, and purification can be found elsewhere.[4] Briefly, the extracellular MHC class II α- and β-chains of H-2A^k, H-2A^{g7}, or H-2E^k were linked via a *fos*/*jun* leucine zipper to the hinge and Fc domain of murine IgG2a. The constructs were transfected into *Drosophila melanogaster* Schneider 2 cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). After hygromycin selection, the *Drosophila melanogaster* Schneider 2 transfectants were grown to a density of 1–2 × 10^7 cells/ml at 22°C and seeded at 3 × 10^6 cells/ml into serum-free medium, and protein production was induced with 500 μM $CuSO_4$. The fusion protein was purified over a protein A column (Repligen, Needham, MA) using a Biologic HR (Bio-Rad, Hercules, CA). After concentration and buffer exchange into PBS/0.05% NaN_3, the fusion protein was adjusted to 2 mg/ml for storage at 4°C. Production and quantitation were monitored by ELISA.

[4] P. Y. Arnold, K. M. Vignali, T. B. Miller, N. L. La Gruta, L. S. Cauley, L. Haynes, P. S. Adams, S. L. Swain, D. L. Woodland, and D. A. A. Vignali. Reliable generation and use of MHC class II:γ2aFc multimers for the identification of antigen-specific CD4$^+$ T cells. *Submitted for publication*.

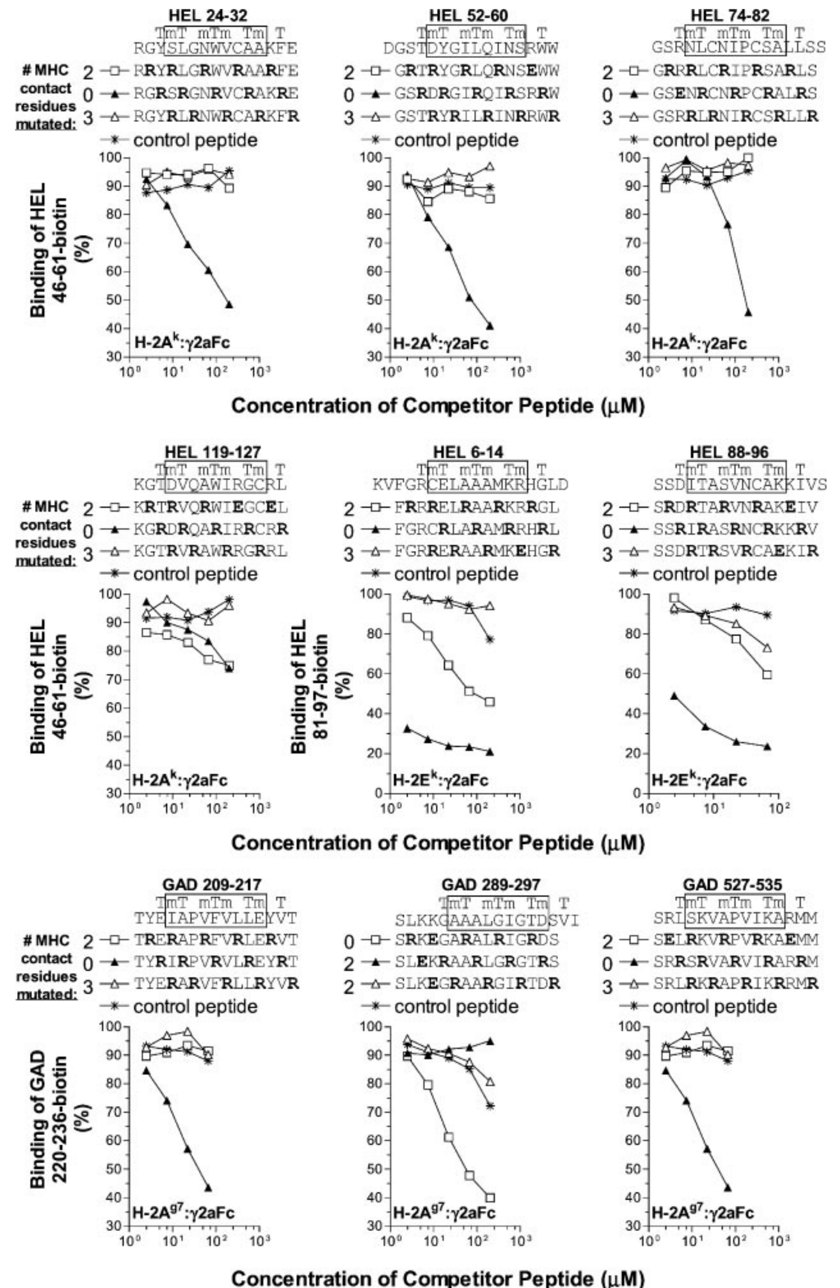

FIGURE 1. Determination of MHC class II:peptide binding register. A competition ELISAwas used to determine the ability of mutant peptides to bind to MHC class II molecules. Biotinylated peptides for H-2A^k:γ2aFc, H-2E^k:γ2aFc, and H-2A^{g7}:γ2aFc were HEL 46–61, HEL 81–97, and GAD 220–236, respectively. Each graph represents the titration of three mutant peptides for each epitope and the mean of two to five experiments. The amino acid sequence of the peptides is listed above each graph with arginine (R)/glutamic acid (E) substitutions in bold. Above the peptides is the native sequence with the register defined by the assay; P1–P9 is boxed and MHC (m) and TCR (T) pointing residues are indicated. Numbers on the *left* of the peptide sequences indicate the number of residues mutated that point toward the MHC (putative contact residues). Nonbinding control peptides are included: HEL 105–119 or HEL 85–100 for H-2A^k:γ2aFc and H-2A^{g7}:γ2aFc, and HEL 48–62 for H-2E^k:γ2aFc.

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Peptides

Wild-type, PFR alanine-substituted, and biotinylated peptides used in functional and peptide binding assays were synthesized on a Rainin Symphony (Woburn, MA) purified by HPLC, and quantified by amino acid analysis. The arginine- and glutamic acid-substituted peptides used for binding register determination were produced by pin synthesis on an ACT 396 Omega (Advanced ChemTech, Louisville, KY) and quantified by absorbance at 210 nm (specific for peptide bonds), using the appropriate wild-type peptide described above as a reference. Amino acid analysis of selected pin peptides demonstrated a concentration variance of no more than 2-fold from the concentration determined by OD. Peptide biotinylation was performed on the resin in two steps: 1) addition of a caproic acid linker (spacer), and 2) addition of the biotin moiety. All peptides were produced by the Hartwell Center, St. Jude Children's Research Hospital (Memphis, TN), and verified using analytical HPLC and mass spectrometry.

MHC class II peptide binding assay

Fusion proteins H-2A^k:γ2aFc (6 μg/ml), H-2E^k:γ2aFc (15 μg/ml), or H-2A^{g7}:γ2aFc (4 μg/ml) were incubated with the appropriate biotinylated peptide, HEL 46–61 (400 nM), HEL 81–97 (2 μM), or GAD 220–236 (500 nM), respectively, and a titration of mutant competitor peptide at the concentrations indicated in McIlvaine citrate phosphate buffer, pH 5 (32), for 24–48 h in 96-well preblocked (5% BSA, TBS Tween 20) plates. Na_2HPO_4 (20 μl, 1 M) and BSA (30 μl, 10%) were added to the reactions and transferred to Immulon 4 HBX ELISA plates (Dynex Technologies, Chantilly, VA) that were previously coated with anti-mouse IgG2a (R11–89; BD PharMingen, San Diego, CA) for 1 h at 37°C, washed, and blocked for 1 h at 37°C. Plates were washed and probed with streptavidin-HRP (1:5000 in 0.05% Tween 20 PBS; Zymed Laboratories, San Francisco, CA), developed with one-step Turbo TMB-ELISA (Pierce, Rockford, IL), and read at 450 nm.

Generation of murine T cell hybridomas

T cell hybridomas were generated as previously described (19). B10.BR and NOD mice (The Jackson Laboratory, Bar Harbor, ME) were used to generate T cell-specific HEL and GAD peptides, respectively. Some fusions were also performed with cells from mice immunized with either HEL protein (Sigma-Aldrich, St. Louis, MO) or rHEL.L56F/W62Y produced in *Pichia pastoris* yeast (29). Preliminary screening involved analysis of CD3 and CD4 expression by flow cytometry and of IL-2 production in response to the immunogen and native protein where appropriate.

Ag presentation assays

Assays were performed essentially as described elsewhere (19, 24, 33). Briefly, T cell hybridomas (5 × 10^4/well) were stimulated with LK35.2 (for HEL-specific hybrids) or M12C3.g7 (for GAD 65-specific hybrids; kindly provided by E. Unanue, Washington University, St. Louis, MO), APCs (2.5 × 10^4/well), and peptides at the concentrations indicated. Synthetic peptides were made using either standard Fmoc chemistry or Chiron pin technology in the Hartwell Center. Purity and quality were verified by HPLC and matrix-assisted laser desorption ionization-time of flight mass spectrometry. Supernatants (100 μl) were removed after 24 h for estimation of IL-2 secretion by culturing with the IL-2-dependent cell line CTLL-2 (1 × 10^4 cells in 100 μl). After 24 h of culture, proliferation was determined by pulsing with [^{3}H]thymidine (1 μCi/well; DuPont Pharmaceuticals, Wilmington, DE) for the final 6 h. These assays were used to determine the concentration of peptide (EC_{50}) required to stimulate a 50% maximal CTLL response.

Flow cytometry

Analysis of T cell hybridomas for CD3 and CD4 expression was performed as described elsewhere (Abs from BD PharMingen) (24, 33).

Lymph node proliferation assays

Assays were performed as previously described (19). Briefly, B10.BR mice (The Jackson Laboratory) were immunized with 14 nmol of HEL, HEL peptide, or mutant peptide as described above. After 7–10 days, lymph node cells were removed and cultured in 96-well flat-bottom plates (6 × 10^5/well) with HEL protein or peptides in complete S-MEM with 10% FCS. Proliferation was measured by pulsing with [^{3}H]thymidine (1 μCi/well) during the last 24 h of a 96-h culture.

Results

Defining MHC class II:peptide binding registers

The identification of PFRs that are recognized by the TCR is dependent on the accurate establishment of the MHC class II peptide binding register for each epitope. The conventional and widely used approach employs truncated peptides or single amino acid substitutions to define MHC binding residues (33–39). However, this does not always lead to definitive answers. Here, we present a new approach using a standard MHC:peptide competition assay with peptides containing five amino acid substitutions at defined positions.

The crystallographic analysis of 14 MHC class II:peptide complexes has clearly shown that the periodicity of peptide binding is essentially conserved (2–13). Thus, P1, P4, P6, and P9 always point toward the MHC, whereas P-1, P2, P5, P8, and P11 always point toward the TCR. Because most of the binding energy between MHC class II molecules and the peptide is usually confined to the peptide backbone, "removal" of a side chain by alanine substitution often has little or no effect on peptide binding. Indeed, some investigators have found that polyalanine (or most residues substituted for alanine) can bind to MHC class II molecules with reasonable affinity (2, 40). However, large bulky and/or charged residues are often not tolerated. We reasoned that substitution of all of the potential TCR binding residues with a large, charged

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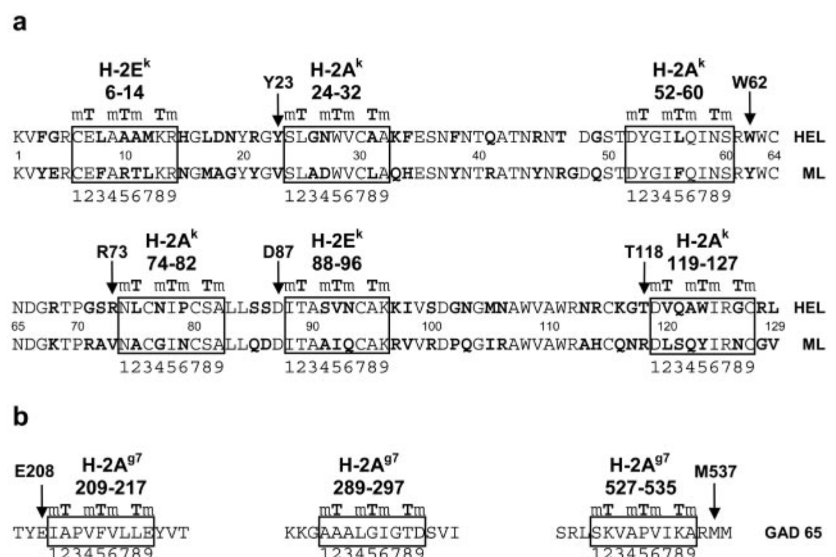

FIGURE 2. PFR recognized by murine T cells specific for HEL or GAD epitopes. *a*, Alignment of HEL and ML (only the predominant ML-M isoform is shown; found in milk, macrophages, and myeloblasts). Residues that differ between HEL and ML are shown in bold. Numbering is for HEL. *b*, Sequence of the three GAD epitopes is shown. *a* and *b*, Boxes outline H-2A^k-, H-2E^k-, and H-2A^{g7}-binding registers defined in Fig. 1 (P1–P9). Residues that could interact with MHC or TCR are indicated (m, MHC binding; T, TCR binding). Numbers below boxes refer to peptide residue position. Arrows indicate PFR recognized by T cell hybridomas generated by immunization with HEL protein or peptides. Determination of PFR dependence is described in Figs. 3 and 5.

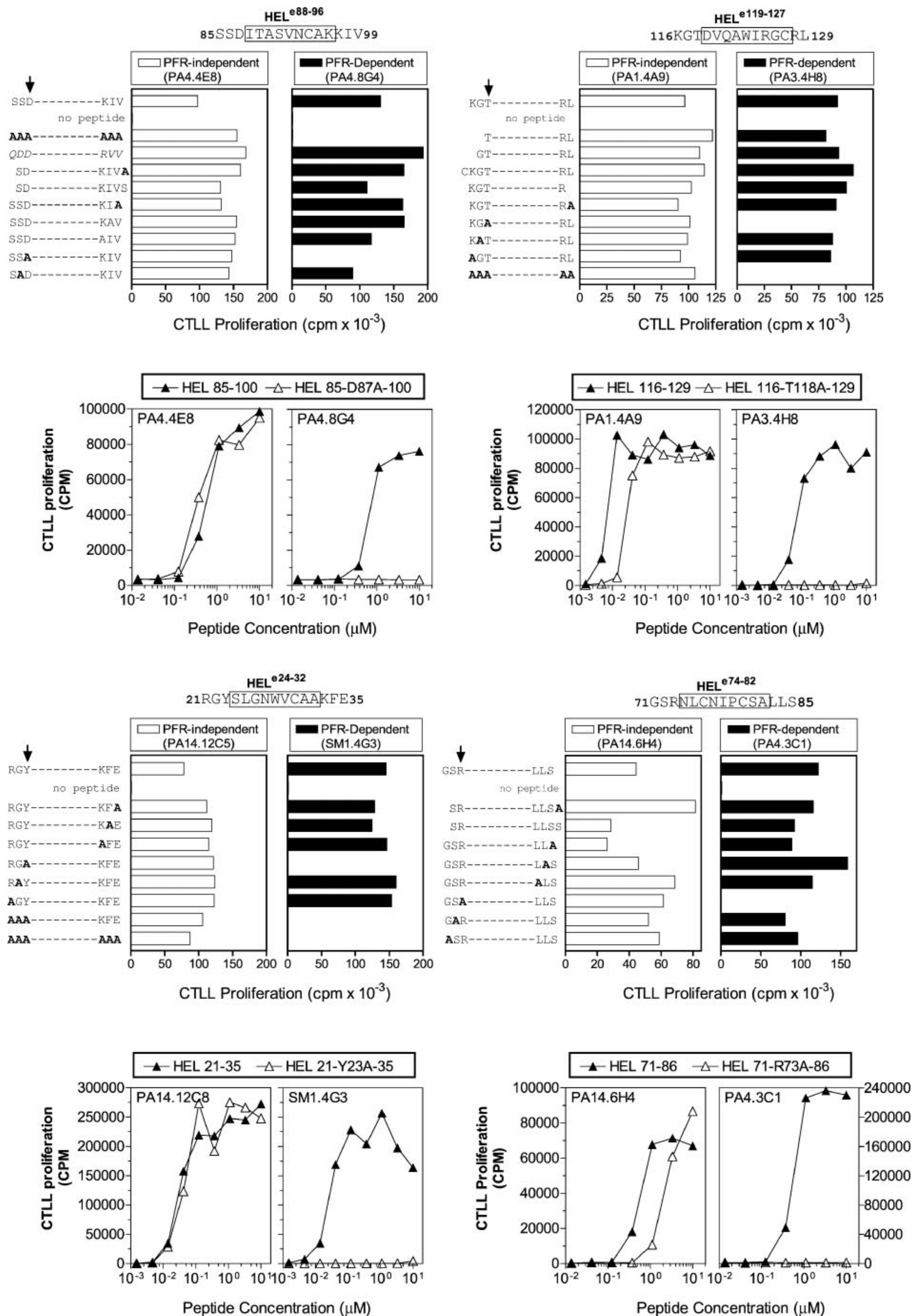

FIGURE 3. Characterization of HEL^{e24-32}, HEL^{e74-82}, HEL^{e88-96}, and HEL$^{e119-127}$-specific, PFR-dependent T cell hybridomas. Data represent IL-2 production, as determined by proliferation of the IL-2-dependent cell line CTLL-2 and by representative PFR-independent (□) and -dependent (■) hybridomas. Bar graphs represent response to 5 μM analog pin peptides presented on the LK35.2 B cell hybridoma. Arrows indicate PFRs recognized by

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Table I. *HEL- and GAD65-specific T cell hybridomas exhibit variable PFR dependency*[a]

Epitope	PFR	Position	Protein			Peptide			Total % Dep[b]
			No. indep	No. dep	% dep	No. indep	No. dep	% Dep	
HEL^{e6-14}			3 (–3)[c]	0	0	8	0	0	0
HEL^{e24-32}	Y23	P-1	7 (5/2)	2 (2/−)	22	ND	ND	ND	22
HEL^{e52-60}	W62	P11	1 (1/−)	5 (5/−)	83	12[d]	22[d]	65	68
HEL$^{74-82}$	R73	P-1	4 (2/2)	20 (4/16)	83	ND	ND	ND	83
HEL^{e88-96}	D87	P-1	7 (−/7)	3 (−/3)	30	30	2	6	12
HEL$^{e119-127}$	T118	P-1	8 (2/6)	5 (2/3)	38	11	20	65	57
GAD$^{e209-217}$	E208	P-1	ND	ND	ND	4	10	71	71
GAD$^{e289-297}$			ND	ND	ND	9	0	0	0
GAD$^{e527-535}$	M537	P11	ND	ND	ND	0	21	100	100

[a] For HEL-specific hybridomas, B10.BR mice were immunized with either protein (HEL or rHEL.L56F/W62Y) or peptide (HEL 1-18, 33-47, 85-100, or 116-129) emulsified in CFA. For GAD-specific hybridomas, NOD mice were immunized with peptide emulsified in IFA. Dependence on PFR was determined as detailed in Figs 3 and 5. All hybridomas are >90% positive for CD4 and CD3 expression.

[b] The number of hybridomas from protein and peptide immunizations was pooled and the total percentage of PFR dependency was determined.

[c] Numbers in parentheses indicate a breakdown of the hybridomas obtained from the wild-type HEL fusion and the rHEL.L56F/W62Y fusion, respectively.

[d] These hybridomas were derived from a previous publication and are included for comparison purposes (19).

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residue, such as arginine or glutamic acid, would not significantly affect peptide binding. However, such substitutions in two or more side chains pointing toward the MHC are likely to disrupt binding.

On this premise, we synthesized three mutant peptides for each epitope for use in an ELISA-based competition peptide binding assay using soluble MHC class II dimers. First, the binding register for each epitope was predicted on the basis of previous structural and functional studies (2–4, 7, 36, 41–46). See Fig. 1 for details of the peptides used. Second, mutant peptides were produced with the spacing x, x + 2, x + 5, x + 8, x + 11, which is the same as that between the TCR-exposed residues at P-1, P2, P5, P8, and P11. One of the three mutant peptides had substitutions at P-1, P2, P5, P8, and P11 for this predicted register, whereas the other two peptides had these substitutions "slid" one amino acid toward the NH_2 or COOH terminus. If the predicted binding register is correct, this would result in the latter two peptides containing two or three substitutions, respectively, of side chains that point toward the MHC. We chose to use arginine as the substituting amino acid due to its size and charge. For peptide residues that were arginine or lysine, glutamic acid was used as the substituting amino acid.

In this study, we used this assay to define the binding register within nine epitopes: four restricted by H-2A^k (HEL 21–35, HEL 48–63, HEL 71–86, and HEL 116–129), two by H-2E^k (HEL 1–18 and HEL 85–100), and three by H-2A^{g7} (GAD 206–220, GAD 284–300, and GAD 524–538) (26, 47, 48). This approach proved to be very effective in unambiguously defining the binding resister (Fig. 1). In almost all cases, only one of the three mutant peptides bound to its respective MHC allele. For certain peptides (HEL 1–18 and HEL 85–100), more than one peptide bound. However, there was one peptide for each epitope that was clearly superior to the other two in the set. The only exception was for HEL 116–129, in which case two of the three peptides bound comparably, albeit weakly. This may have been due to the weak binding of these peptides and should be taken into account when assessing data presented using this epitope. All of our initial predictions were correct with the exception of GAD 284–300. Two potential registers were proposed (2), and we chose to make peptides for the NH_2-terminal register. However, our data clearly show that the COOH-terminal register is in fact correct.

These experiments allowed us to define the epitopes within the nine peptides examined as HEL^{e24-32}, HEL^{e52-60}, HEL^{e74-82}, HEL$^{e119-127}$, HEL^{e6-14}, HEL^{e88-96}, GAD$^{e209-217}$, GAD$^{e289-297}$, and GAD$^{e527-535}$ (Fig. 2). To avoid confusion between core epitopes and synthetic peptide sequences in this study, core MHC class II binding epitopes (P1–P9) will be denoted by the suffix "e" and superscripted (e.g., HEL^{e52-60}). Synthetic peptides will be noted in the conventional way (e.g., HEL 48–63).

PFRs are recognized by T cells specific for HEL and GAD 65 epitopes

We have previously shown that T cell hybridomas generated against HEL^{e52-60} were predominantly dependent on the W62 PFR (19). To study T cell dependence on PFRs further, we generated T cell hybridomas specific for six HEL epitopes defined above by immunization with HEL protein or peptides. Only T cell hybridomas (>90% $CD4^+CD3^+$; 130 in total) that responded in vitro to the native HEL protein (as measured by IL-2 production) were analyzed further. PFR dependency was determined using a panel of peptides containing alanine substitutions at PFRs. PFR-independent hybridomas were defined by their ability to respond to all of the peptides, whereas PFR-dependent hybridomas responded to all peptides except those that contained substitutions at a single PFR. PFR-dependent T cell hybridomas (40% of total) were generated against all of the H-2A^k- and H-2E^k-restricted epitopes studied, except for HEL^{e6-14}, which only generated 11 hybridomas (Table I). The percentage of T cell hybridomas that were dependent on a particular PFR varied from 12 to 83%.

Analysis of T cells specific for the H-2A^k-restricted epitopes HEL^{e24-32}, HEL^{e74-82}, and HEL$^{e119-127}$ and the H-2E^k-restricted epitope HEL^{e88-96} revealed dependency on residues at the P-1 position (Y23, R73, T118, and D87) (Fig. 3). Importantly, PFR-dependent T cell hybridomas were obtained from mice immunized with HEL protein, indicating that PFR-dependent T cells are generated from naturally processed peptides. In almost all cases, PFR

dependent hybrids. Bold type indicates alanine substitutions and italic type indicates substitution with comparable ML residues. All peptides contain the core nine-residue MHC class II-binding sequence of the particular HEL epitopes detailed in Fig. 2 and boxed in sequence above bar graph (indicated by hyphens). Line graphs indicate PFR dependence as demonstrated by an inability to respond to purified mutant peptide (HEL 21-Y23A-35, 71-R73–86, 85-D87A-100, and 116-T118A-129) in which the specific PFRs have been substituted for alanine. Comparable responsiveness of PFR-independent T cells to both peptides also indicates that substitutions do not affect peptide binding.

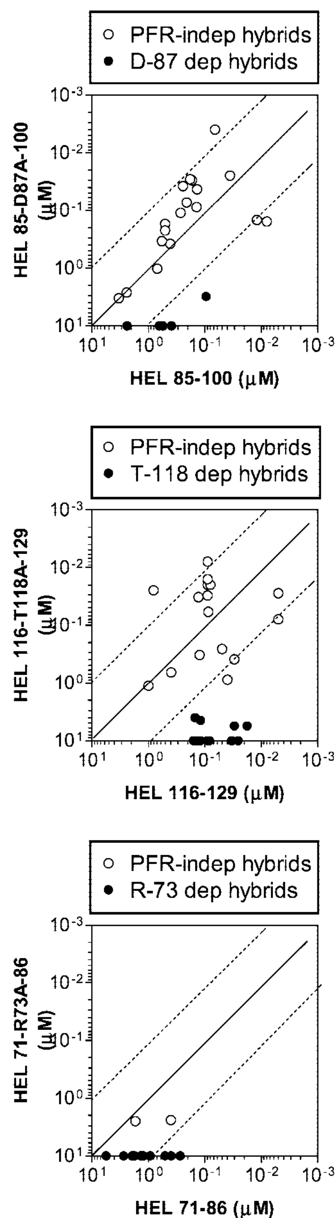

FIGURE 4. PFR-independent and -dependent T cells respond with comparable sensitivity to wild-type peptides. Each symbol represents one T cell hybridoma (○, PFR independent; ●, PFR dependent). Hybridomas are plotted according to the response (EC_{50}, concentration of peptide required to give 50% maximal IL-2 production) to wild-type peptide (*x*-axis) vs the response to analog peptides where the PFR is substituted with alanine (*y*-axis). The diagonal solid lines indicate where EC_{50} values for both wild-type and mutant proteins are identical. The dashed lines are 1 $\log_{10}$ to either side of the solid line. Hybridomas near to or within the dashed lines are considered PFR independent.

dependency was absolute in that T cell hybridomas did not respond to the mutant peptides containing the PFR alanine substitutions even at the highest concentration, but they responded strongly to the wild-type peptide (Figs. 3 and 4 and data not shown). In contrast, PFR-independent hybridomas responded comparably to both peptides. Dependence on PFR was not a consequence of reduced sensitivity to Ag, because PFR-dependent and -independent T cells responded with equal sensitivities to most wild-type peptides (Fig. 4). The exception was HEL^{e88-96}-specific T cells dependent on D87. It is possible that tolerance induction toward this residue, which is conserved between HEL and murine lysozyme (ML), may limit the sensitivity of these PFR-dependent T cells.

We extended these studies further to examining T cell responses to the major autoantigen involved in the initiation of type 1 diabetes, GAD 65 (48, 49). We generated and analyzed 44 T cell hybridomas to $GAD^{e209-217}$, $GAD^{e289-297}$, and $GAD^{e527-535}$ by peptide immunization of NOD mice. PFR-dependent T cell hybridomas were again identified, with those specific for $GAD^{e209-217}$ requiring the P-1 residue, E208, whereas all of the hybridomas generated to $GAD^{e527-535}$ were dependent on the P11 residue M537 (Table I and Fig. 5). Titration analysis showed that the PFR-independent T cell hybridomas specific for $GAD^{e209-217}$ responded equally to GAD 206–220 and GAD 206-E208A-220 (data not shown). All of the $GAD^{e289-297}$-specific T cell hybridomas generated were PFR-independent. It is noteworthy that the four P-1/P11 residues to which PFR dependency was not observed were either small or hydrophobic amino acids (V219, G288, V299, and L526).

Importantly, alanine substitution of the PFR had only a modest effect on peptide affinity in that both the wild-type and mutant peptides bound to their respective MHC class II molecules with similar affinity (Fig. 6). Although HEL 71-R73A-86 bound with slightly reduced affinity, this only had a small effect on the ability of PFR-independent T cells to respond to the mutant vs wild-type peptides (Fig. 3 and data not presented), suggesting that this cannot explain the inability of PFR-dependent T cells to respond to the mutant peptides.

In summary, seven of the nine HEL and GAD epitopes studied, restricted by three different MHC class II molecules, were found to generate varying degrees of PFR dependency (12–100%) that was directed exclusively to the P-1 or P11 position. It was also evident that PFR dependency was more frequently found toward residues at P-1 than toward those at P11 (five vs two, respectively; this study and Ref. 19).

PFRs potentiate T cell responses and contribute to peptide immunogenicity

Previous studies in our laboratory have demonstrated that the PFR W62 significantly enhances the immunogenicity of the HEL 48–63 peptide (19). Therefore, we evaluated whether PFR recognition always leads to increased immunogenicity. First, we determined the immunodominance hierarchy of the various HEL peptides by in vitro restimulation of lymph node T cells from B10.BR mice ($H\text{-}2^k$) immunized with HEL protein (Fig. 7). The T cell response was dominated by the $H\text{-}2A^k$-restricted epitopes HEL^{e24-32}, $HEL^{e119-127}$, and HEL^{e52-60}, all of which generated a significant percentage of PFR dependency (22, 57, and 68%, respectively; see Table I). In contrast, most of the weaker epitopes generated no or low percentages of PFR-dependent T cell hybridomas (0–12%), with the exception of the HEL^{e74-82} (83%). It is noteworthy that although the lymph node proliferation response to this peptide was weak, a significant percentage of the hybridomas generated from the HEL fusion were specific for this epitope (37%). Thus, there may be a relationship between PFR dependency and immunodominance.

T cells from mice immunized with HEL 48–63 exhibited a stronger in vitro recall response to the native HEL protein compared with mice immunized with peptide lacking the W62 PFR (19). To determine whether PFR recognition always contributes immunogenicity, we immunized B10.BR mice with HEL protein, wild-type peptide, or mutant peptide containing an alanine substitution at the PFR previously shown to mediate dependency. Lymph node cells from immunized mice were restimulated in vitro with all three immunogens. As previously shown for the

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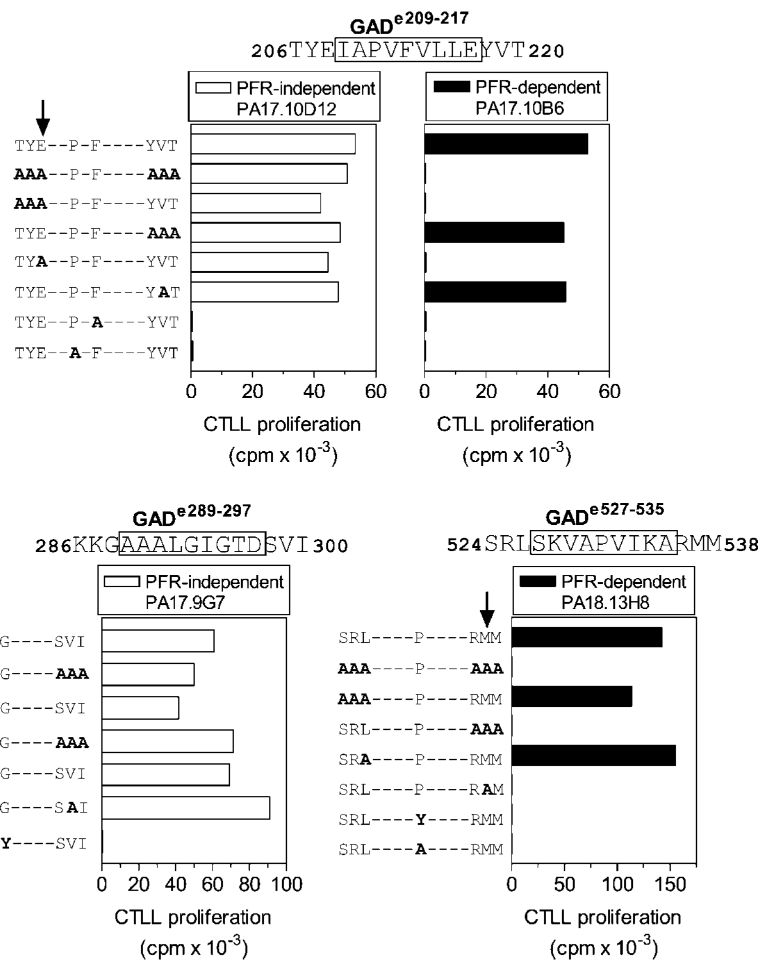

FIGURE 5. T cell dependence on PFRs of GAD 65 epitopes. PFR-independent (□) and -dependent (■) hybridomas were generated from NOD mice immunized with a mixture of GAD peptides (206–220, 284–300, and 524–538) emulsified in IFA. Data represent IL-2 production by hybridomas in response to 2 μM analog pin peptides presented on the M12C3.g7 B cell hybridoma. Arrows indicate PFRs recognized by dependent hybrids. All peptides contain the core nine-residue MHC class II-binding sequence of the particular GAD epitopes as boxed in sequence above bar graph (indicated by hyphens). PFR dependence is demonstrated by inability to respond to mutant peptides (GAD 206-E208A-220 and 524-M537A-538) in which PFRs have been substituted for alanine.

HEL^{e52-60} epitope, wild-type peptides were significantly more immunogenic than were the alanine mutant peptides (HEL 21-Y23A-35, HEL 85-D87A-100, and HEL 116-T118A-129) (Fig. 8; compare ○ in the *center graphs* with ▲ in the *right graphs*). Indeed, wild-type peptides derived from the epitopes that generated PFR dependency were also more immunogenic than those that did not (e.g., HEL 1–18) (Fig. 8; compare ○ in the *center graphs*). The most striking observation was that two of the epitopes that generated the greatest percentage of PFR-dependent T cell hybridomas (HEL^{e24-32} and HEL$^{e119-127}$) also generated a much stronger recall response to the native HEL protein, compared with epitopes that generated few or no PFR-dependent T cells (HEL^{e88-96} and HEL^{e6-14}, respectively) (Fig. 8; compare ■ (HEL) in the *center graphs*). In contrast, peptides lacking critical PFRs were generally weak immunogens, inducing a poor in vitro recall response to full-length and mutant peptides as well as to native HEL. The relatively weak recall response to HEL after immunization with HEL 85–100 is consistent with the low percentage of T cell hybridomas dependent on D87 (6%), which may be due to tolerance induced by this HEL:ML conserved residue (Fig. 2). The only exception to this general finding was HEL^{e74-82}, which generates a high percentage of PFR dependency but is a weak immunogen (compare Table I with Fig. 7). Taken together, these results demonstrate that T cell recognition of PFRs is an important factor that contributes to peptide immunogenicity and may influence immunodominance.

Discussion

We have previously shown that the majority of HEL 48–63-specific T cells are dependent on the COOH-terminal PFR W62 (19). We have now examined the PFR dependency of 174 new T cell hybridomas generated to nine immunogenic epitopes from HEL and GAD presented by three MHC class II molecules. Of these nine epitopes, seven were recognized by PFR-dependent T cells (40% of total hybridomas). We also have preliminary data suggesting that PFR-dependent T cells are generated to H-2A^{d}:OVA$^{e324-332}$ and H-2A^{b}:sHN$^{e421-429}$ (Sendai virus hemagglutinin-neuraminidase). Interestingly, the only positions that generated PFR dependency were P-1 and P11, which is consistent with the notion that all peptides bind to MHC class II molecules with a common periodicity, such that residues at P-1 and P11 are the PFRs most likely to be accessible to the TCR (2–5, 7–9). However, it is also evident that P-1 is favored over P11. Of the seven amino acids that mediated PFR dependency, five were in the P-1 position, whereas two were at P11 (this study and Ref. 19). Preferential recognition of P-1 may be due to a favored interaction of the TCR with the NH_2-terminal region of the MHC:peptide complex (49, 50). Therefore, we can reasonably predict that the majority of immunogenic epitopes presented by MHC class II molecules will generate PFR-dependent T cells and that this recognition would be restricted to P-1 and/or P11. Given that the epitopes studied derive from two different proteins and are restricted by three MHC class II molecules, we propose that PFR recognition is a common event characteristic of MHC class II-restricted T cell responses. Furthermore, PFR recognition by T cells is clearly a feature of MHC class II:peptide complexes that could not be used in TCR recognition of MHC class I-restricted epitopes.

Is there another explanation for our data other than direct recognition of PFRs by the TCR? It has been suggested that some

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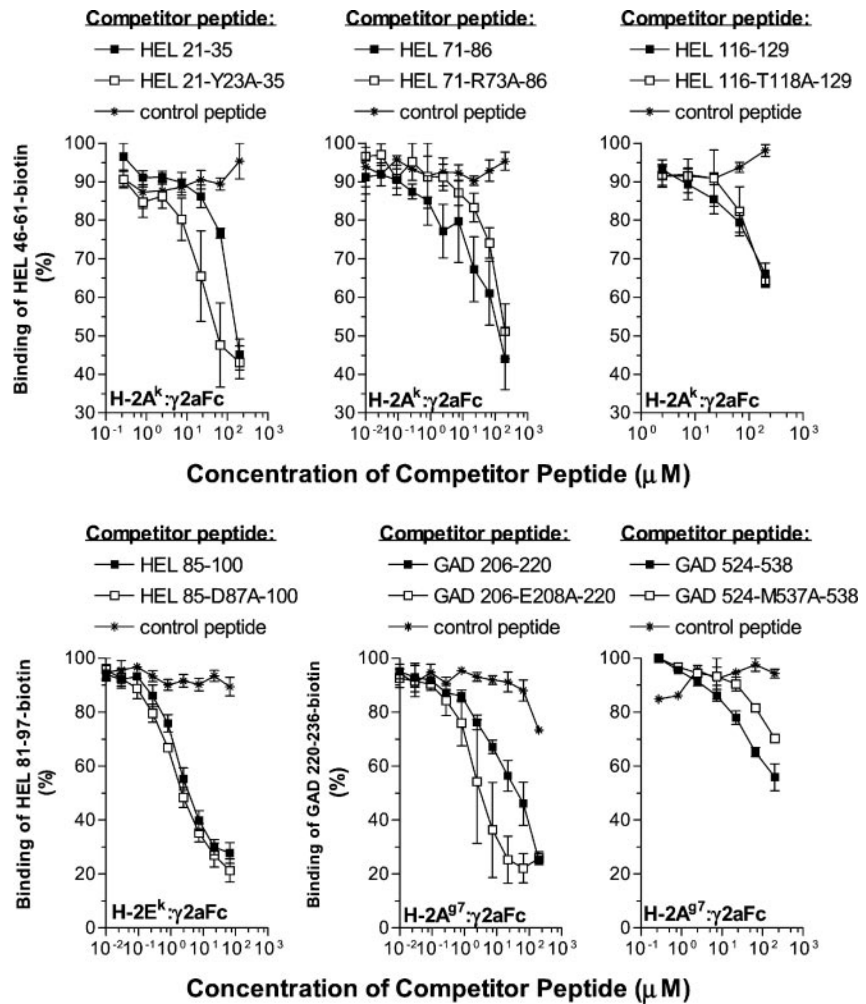

FIGURE 6. PFR substitutions have little effect on peptide/MHC class II binding. Alanine-substituted peptides were titrated at the indicated concentrations and mixed with the appropriate MHC class II:Ig fusion protein and biotinylated peptide in a competition ELISA. Biotinylated and control peptides were detailed in Fig. 1. Data represent the mean of two to five experiments ± SD.

peptides may bind in two alternate registers (i.e., the assignment of the P1 residues may shift by one or two residues either way) (2, 5). Although this is theoretically possible, no crystallographic data have been presented to directly support this idea. Indeed, H-2A^{g7}-GAD 65 221–235 has been speculated to bind with the core sequence of 224–232 or 226–234. However, only 224–232 was observed at P1–P9 in the crystal structure (2). Likewise, H-2A^d-OVA 323–339 may bind with the core sequence of 324–332, 329–337, or 326–334, but only 324–332 was observed at P1–P9 in the structure (5). Our binding experiments clearly showed that all but one epitope bound in a single register. The only exception was HEL$^{e119-127}$, which may bind in two registers. However, because these peptides bound poorly, an unequivocal answer could not be obtained. There are two further reasons why peptides are unlikely to bind in alternate registers or that PFR substitutions alter this register. First, if PFR substitution alters peptide binding register, then PFR-independent hybridomas would also fail to respond to mutant peptides because the key residue at P5 would no longer be accessible. Second, if some of the register assignments were incorrect, it is unlikely that only P-1 and P11 substitutions would have affected T cell function. For example, a single amino acid shift to the right in the register would move the residue in the P-1 position to P-2 and P11 to P10. This is not evident from our studies because we did not generate hybrids dependent on PFRs in other positions (P-3, P-2, P10, and P12 were analyzed in this study).

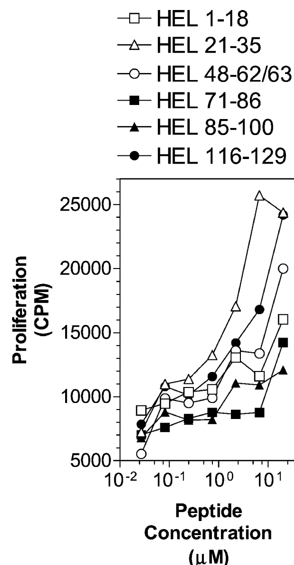

FIGURE 7. Hierarchy of HEL peptide immunodominance. Lymph node cells from B10.BR mice immunized with HEL/CFA were stimulated in vitro with various HEL peptides. Cultures were pulsed during the last 24 h of a 96-h assay. Data are means of four separate experiments.

What factors influence whether residues at P-1 and P11 mediate PFR dependency? There are three possibilities: 1) the type of amino acid, 2) tolerance induced by the murine homologue/

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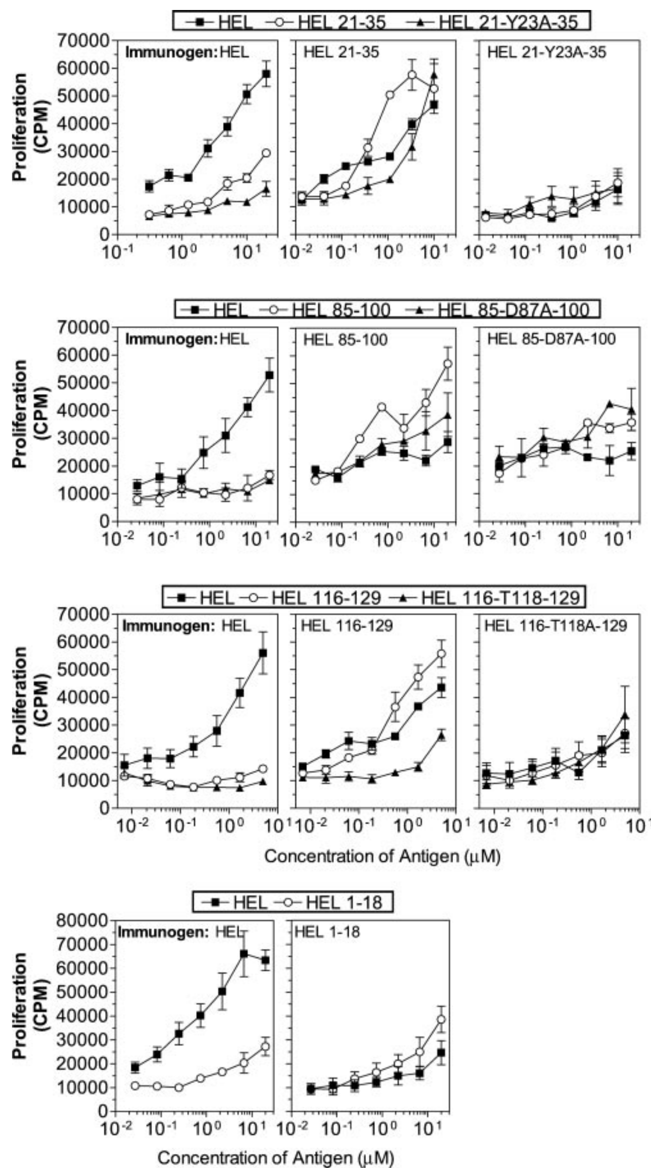

FIGURE 8. PFRs enhance peptide immunogenicity. B10.BR mice were immunized with HEL (*left graphs*), wild-type HEL peptides containing PFRs (*middle graphs*), and HEL peptides in which relevant PFRs are substituted for an alanine (*right graphs*). Lymph node cells were collected and restimulated with HEL, wild-type, and mutant peptides at the concentrations indicated. Each graph represents the mean proliferative response of four to six mice from two to three independent experiments.

molecule, and 3) Ag processing. Interestingly, most of the residues at P-1 and P11 that do mediate PFR dependency are capable of forming either a salt bridge or hydrogen bond (Y, W, R, D, T, and E; Fig. 2 and Table II). The one exception is a methionine (GAD.M537). In contrast, most of the residues that did not mediate PFR dependency were small and/or hydrophobic (G, F, V, L, and I). Such residues may not mediate as strong an interaction with the TCR, which may be required for PFR dependency. One caveat, however, involves our use of alanine substitutions to identify residues that mediate PFR dependency. For some residues, alanine may be considered a conservative substitution that fails to abrogate PFR dependency. A similar analysis using peptides with different amino acid substitutions at PFRs would have to be performed to rule out this possibility. Second, tolerance induced by the murine homologue/molecule may also influence the generation of PFR-

Table II. *Summary of PFRs and their ability to mediate T cell dependency*[a]

PFR Dependency			
Yes		No	
P-1	P11	P-1	P11
Y R	W	R[b]	G[b] F
T E	M	T[b]	V V
D[b]		L	L L
		G	I

[a] All of the residues at the P-1 or P11 positions of the six HEL and three GAD epitopes are segregated according to whether they generated PFR-dependent T cells.
[b] These residues are conserved between HEL and the murine homolog.

dependent T cells. We found PFR dependency on the P-1 residues R73 and T118 (which are unique to HEL), yet we found no PFR dependency on the P-1 residues R5 and T51 (which are conserved between HEL and ML) (see Fig. 2). The only conserved HEL:ML residue that did mediate PFR dependency was D87, although only four hybridomas were identified and these were relatively insensitive (see Fig. 4). Lastly, efficient Ag processing may limit the proportion of naturally processed peptides that contain the P-1 or P11 residues (17, 19, 29). However, peptide elution studies performed to date suggest that efficient processing is unlikely to result in all the naturally processed peptides lacking either the P-1 or P11 residues (14–18).

How does the TCR recognize PFR? In addition to the accessibility of different PFRs to the TCR, there may also be structural constraints on the TCR that govern its ability to interact with these residues. For instance, none of the 83 PFR-dependent T cell hybridomas analyzed in this study were dependent on both P-1 and P11. Indeed, analysis of the two TCR:MHC class II structures solved to date suggests that it may be difficult for the TCR to engage both P-1 and P11 at the same time (49, 50). In both structures, direct TCR interaction with P-1 was observed. Additional structures will be required to determine how the TCR recognizes residues at P11.

One of the most striking features of PFR dependency is the fact that it is absolute. All but 7 of the 105 PFR-dependent T cell hybridomas (93%) examined to date completely failed to respond to the maximal concentration (10 μM) of peptide containing the relevant PFR substitution (this study and Ref. 19). Indeed, we have previously shown that PFR-dependent T cells will not even respond to B cells that express a single recombinant MHC:peptide complex, which increases the effective ligand density 100-fold (19). Why does the absence of the appropriate PFR have such a profound affect on T cell function? One possible explanation is that an unusually large proportion of the binding energy is directed toward this residue. The TCR-VαVβ domains are constrained by their requirement to interact productively with the MHC molecule. Therefore, TCR selection of the optimal TCR VαVβ combination for recognition of P-1/P11 residues may seem more difficult than for selection of the best CDR3$\alpha\beta$ to interact optimally with the peptide P5 residue. However, Ding et al. (51) have shown that different TCRs recognizing the same MHC:peptide complex can interact with almost completely different residues, suggesting that there is great plasticity in TCR:MHC recognition. Thus, the TCR repertoire may select the ideal V region to interact with a particular PFR without compromising its ability to bind MHC. This idea would be consistent with the profound restriction of TCR-Vβ usage observed among PFR-dependent T cells (19). Additional structural and functional studies will be required to resolve this important issue.

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In this study, we have clearly shown that PFR recognition has a significant effect on immunogenicity and thus may constitute an important physiological role for PFRs. This is evident from a direct comparison of the immunogenicity of wild-type and mutant peptides and from the strength of the recall response to peptides after HEL immunization or in response to HEL after peptide immunization. Although five of the six H-2A/E^{k}-restricted, HEL-specific epitopes generated PFR-dependent T cells, their proportion varied (12–83%). Indeed, the contribution afforded by PFR dependency to the immune response can be seen most clearly by comparing epitopes that generated high and low numbers of PFR-dependent T cells. There was a good correlation between the percentage of T cell hybridomas that were PFR-dependent and the consequence of PFR substitution on diminution of the T cell response to HEL protein or peptides (see Table I and Fig. 8).

How could PFR dependency contribute to immunodominance? Although this is difficult to assess conclusively from our data, it is evident that the epitopes that generated the largest recall response after immunization with HEL protein (HEL^{e24-32}, HEL^{e52-60}, and HEL$^{e119-127}$) all generate significant percentages of PFR-dependent T cell hybridomas (22, 68, and 57%, respectively; see Table I). This is in contrast with most of the weaker epitopes (0–12%). The only exception was HEL^{e74-82}, which induces a weak T cell response after HEL immunization, yet 83% of the HEL^{e74-82}-specific hybridomas were PFR dependent. These data are consistent with previous studies suggesting that the recall response to this epitope is weak/absent in the absence of R73 (26). It is possible that this epitope is poorly processed or binds weakly to H-2A^{k} and is thus a poor immunogen as part of the native protein. The very high percentage of PFR-dependent T cells observed with this epitope may be due to the composition of the available TCR contact residues because the crucial P5 residue and P8 are conserved between HEL and ML, and P2 and P11 are leucines. This may make the nonconserved, charged arginine residue at P-1 particularly "attractive" to the TCR repertoire. An accurate assessment of the contribution afforded by PFR dependency to immunodominance using MHC class II:peptide tetramers or analogous staining reagents could prove enlightening and is worthy of further investigation.

In summary, our data support the idea that PFR dependency is a common characteristic of MHC class II-restricted T cell responses. Indeed, a number of studies have presented data consistent with PFR dependency (23, 52–57). Interestingly, most of these studies have involved the analysis of human T cell clones, suggesting that PFR dependency is not restricted to the mouse. We would speculate that the TCR repertoire is capable of recognizing the P-1 and P11 residues in any immunogenic epitope, whereby recognition is limited by the type of amino acid tolerance exerted to the host epitope and Ag processing. With this information, we may be able to predict whether PFR recognition is likely to occur for other T cell epitopes. It is particularly interesting that PFR dependency may be one of the most prominent and significant characteristics distinguishing TCR recognition of peptide-bound MHC class I and class II molecules. How might PFR dependency be relevant to MHC class II-restricted T cell responses? Given the large number of peptide residues in MHC class II:peptide complexes that are accessible to the TCR, it is possible that this results in an increased initial precursor frequency. Alternatively, we have previously speculated that PFR may play an immunomodulatory role by regulating the response of PFR-dependent T cells (29). It is also possible that the increased number of TCR:peptide vs TCR:MHC contacts may influence the efficiency of positive and negative selection for MHC class II-restricted T cells. Although the physiological benefit has yet to be fully elucidated, it is clear that PFR dependency significantly contributes to overall immunogenicity and may influence the immunodominance hierarchy.

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