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Fine Structure of a Virus-Encoded Helper T-Cell Epitope Expressed on FBL-3 Tumor Cells

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Antigen peptide f_{n20} representing Friend murine leukemia virus $env_{122-141}$ (DEPLTSLTPRCNTAWNRLKL) is recognized by two independent Friend virus-induced, FBL-3 tumor-specific helper T-cell (Th) clones. We isolated more Th clones from mice immunized with f_{n20} peptide. We examined the fine structure of the peptide required to activate a large group of f_{n20}-specific Th clones. A systematic analysis of peptides of decreasing lengths eliciting Th proliferation defined the minimum core length as 13 amino acids (LTSLTPRCNTAWN). Functional proliferation and competition assays with variant peptides with alanine substitutions permitted the assignment of five peptide residues in two major histocompatibility complex-interacting and three T-cell-receptor-interacting sites. Th clones were different in their reactivities toward peptides of various lengths and the variant peptides.

Immunization of mice with a syngeneic Friend murine leukemia virus (F-MuLV)-induced tumor (FBL-3) elicited the proliferation of potent effector T-cell populations (5, 8). Most cells in the populations were cytotoxic T cells with a $CD8^+$ phenotype, while $CD4^+$ T cells were isolated at a very low frequency (less than 1 in 500) (6). Among them, four helper T-cell (Th) clones were isolated. The localization of these Th determinants on viral proteins was analyzed with recombinant vaccinia viruses expressing F-MuLV *env* or shorter fragments of the *env* gene. Two clones recognized antigen peptide f_{n20} (n20 indicates number of amino acids) representing F-MuLV $env_{122-141}$ (DEPLTSLTPRCNTAWNRLKL). The f_{n20} peptide induced significant protective immunity against Friend virus infection (unpublished data). This finding encouraged us to isolate more Th clones that recognize the $env_{122-141}$ peptide antigen after immunization of mice with the synthetic peptide.

To understand peptide-major histocompatibility complex (MHC) interactions and the subsequent recognition of peptides by specific T cells, we used peptides of decreasing lengths to determine the preferred size of the peptide and variant peptides with single alanine substitutions to identify key amino acids required for binding to the MHC molecule and for stimulating the proliferation of Th clones in vitro.

Most studies analyzing the molecular structure of the class II-restricted antigen peptide have been performed with Th hybridomas, probably because of the difficulty in establishing Th clones in those systems (1, 4, 7, 13). In this study, we showed that a single *env* peptide can be recognized by multiple Th clones with different reactivities toward peptides of various lengths and amino acid residues.

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MATERIALS AND METHODS

Peptide synthesis. We synthesized peptides as described previously (6). In brief, peptides were synthesized by anchoring each C-terminal amino acid on Wang resin by the 9-fluorenylmethoxycarbonyl-based solid-phase method with a model 430A peptide synthesizer (Applied Biosystems Inc., Foster City, Calif.) (3). Each peptide-resin was treated with trifluoroacetic acid–thioanisole–*m*-cresol–ethanedithiol–H_2O (80:5:5:5:5, vol/vol) at room temperature for 2.5 h, and then filtered. The filtrate was precipitated with ether, and the resulting precipitate was collected by centrifugation. The precipitate was dissolved in 1 N acetic acid. Each crude peptide was subjected to high-performance liquid chromatography on a C_{18} reverse-phase column and eluted with a gradient of CH_3CN in aqueous 0.1% trifluoroacetic acid. The desired peak fractions were combined, and the solvent was removed by lyophilization. The predicted composition of amino acids was confirmed by amino acid analysis.

Isolation of peptide-primed Th clones. C57BL/6 mice received subcutaneous injections at the base of the tail and in the footpads of 10 or 100 μg of f_{n20} peptide dissolved in 100 μl of saline and mixed 1:1 with complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) in an emulsion. After 10 days, we prepared lymphocytes from lymph nodes and enriched T cells by passing them through a nylon wool column. T cells (4×10^6) were incubated in complete medium for 7 days at 37°C in 5% CO_2 and air together with 2×10^6 irradiated (20 Gy) syngeneic spleen cells with f_{n20} peptide (0.5 or 5 μg/ml). Irradiated spleen cells served as antigen-presenting cells. Complete medium was RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% calf serum, 5×10^{-5} M 2-mercaptoethanol, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Limiting dilution cultures (2 or 20 cells per well) were mixed with antigen-presenting cells in the presence of f_{n20} peptide (0.5 or 5 μg/ml) and human recombinant interleukin-2 (TGP-3; Takeda Pharmaceutical Industries Ltd., Osaka, Japan) (2.5 ng/ml). Growing colonies were screened by flow cytometry for surface expression of CD4. All the $CD4^+$ T-cell clones from the limiting dilution cultures were expanded and

tested to determine whether they specifically responded to syngeneic spleen cells with f_{n20} peptide. Those Th clones which responded to the cells with f_{n20} were recloned on a 0.5-cell-per-well basis and passaged every 7 days together with antigen-presenting cells with f_{n20} peptide (0.5 or 5 μg/ml) in the presence of human interleukin-2.

Proliferation assay. Proliferation in triplicate cultures in wells of flat-bottomed microculture plates was measured. Irradiated (20 Gy) spleen cells (5×10^5) and various concentrations of a synthetic peptide were mixed with 1×10^5 cloned $CD4^+$ T cells in a total volume of 200 μl of complete medium in each well without human interleukin-2. The plates were incubated at 37°C for 48 h, and 18.5 kBq of [^{3}H]thymidine (74 GBq/mmol; Amersham/Seale, Arlington, Ill.) was added to each well 4 h before the termination of the culture. The cells were then collected onto a glass fiber filter, and radioactivity was measured with a liquid scintillation spectrometer. All the data for the triplicate cultures are expressed in the figures as mean counts per minute ± standard deviations.

RESULTS

Effect of peptide length on activation of Th clones. Two $CD4^+$ Th clones, SB14-31 and BL4L-23, were isolated from mice immune to a Friend virus-induced tumor, FBL-3 (6). These two clones were specific for the f_{n20} peptide representing the F-MuLV $env_{122-141}$ sequence (DEPLTSLTPRCN TAWNRLKL) bound to the MHC class II I-A^b molecule. In this study, we isolated more Th clones from mice immunized with f_{n20} peptide. Th clones from the BP1 and BP2 series were isolated from mice primed with 10 μg of f_{n20} peptide, and Th clones from the BP3 series were from mice primed with 100 μg of the peptide. The concentrations of f_{n20} used for primary culture, limiting dilution culture, and passage were 0.5 μg/ml (0.21 μM) for BP2 and BP3 and 5 μg/ml (2.15 μM) for BP1.

T-cell activation was assayed by cell proliferation (results are shown in Fig. 1), and the peptide concentration required for half-maximal proliferation (ED_{50}) was measured (Table 1). Group I clones, which are represented by SB14-31, showed a high sensitivity for f_{n20}, with an ED_{50} of 0.01 μM. Other clones showed increasing ED_{50}s: 0.1 to 0.3 μM for group II (BL4L-23 and BP2-6), 0.9 to 1.1 μM for group III (BP3-7 and BP3-13), and 17 μM for group IV (BP1-2 and BP1-3).

To determine the essential sequence of the f_{n20} peptide, we made truncations of the peptide and tested their abilities to activate different Th clones. Four C-terminal residues were removed to obtain $f_{n16(122-137)}$, and an additional three N-terminal residues were removed to obtain $f_{n13(125-137)}$. The specific activity of each test peptide for each representative clone is expressed as the ratio of the ED_{50} of f_{n20} to that of the test peptide (Table 1). Truncations of the peptides reduced the proliferation activity of each clone to various degrees. Three clones, BP2-6 (group II) and BP1-2 and BP1-3 (group IV), were virtually unresponsive to f_{n16} peptide. All groups also failed to respond to f_{n13}. The losses of activity were 10-fold for group I, more than 100-fold for group II, and more than 10-fold for groups III and IV.

To show that proper peptide length is a requirement for activation, the f_{n13} peptide was elongated to 20 residues with polyalanines (f_{n13A7}) which have small interactions over many peptide side chains. Peptide f_{n13A7} significantly increased the activity of Th clones showing marginal activity with the f_{n13} peptide (Fig. 1 and Table 1). The data show that the f_{n13} peptide may contain key MHC and T-cell-receptor (TCR) residues but that the proper length of 20 residues is required for stable TCR-peptide contacts or formation of stable peptide-MHC complexes.

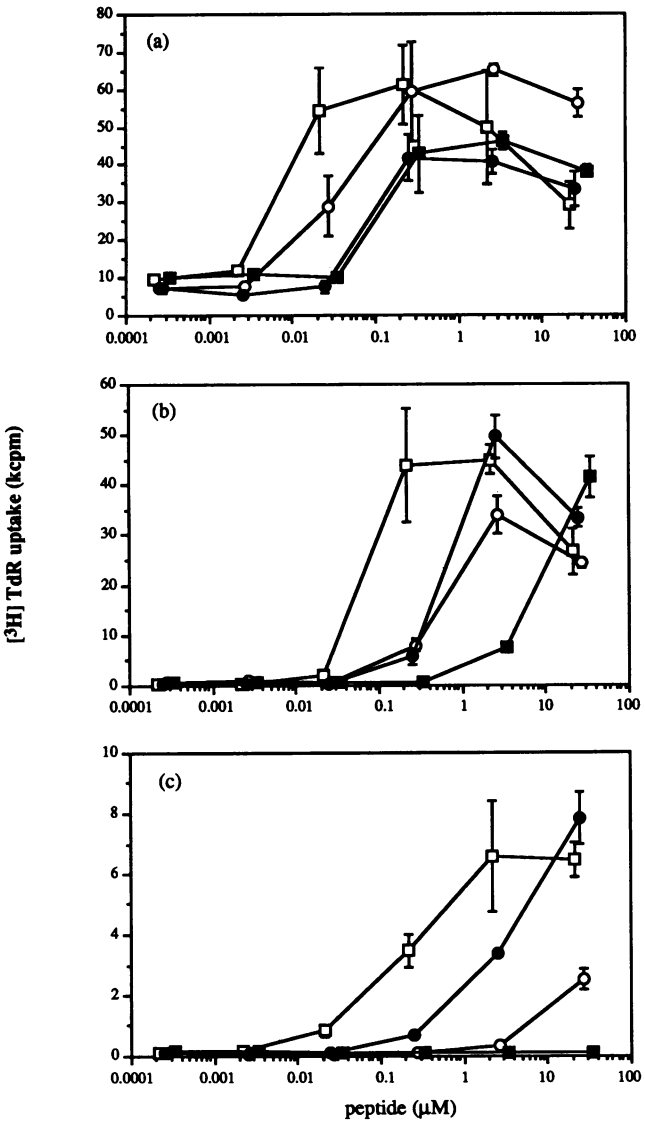

FIG. 1. Proliferation of Th clones SB14-31 (a), BL4L-23 (b), and BP2-6 (c) stimulated by peptides of various lengths. f_{n20} (□), f_{n16} (○), f_{n13} (■), and f_{n13A7} (●) were added to Th cells in the presence of irradiated syngeneic spleen cells without interleukin-2. Error bars show standard deviations. [^{3}H]TdR, [^{3}H]thymidine.

Critical alanine substitutions in peptides recognized by Th clones. Since key MHC and TCR residues may be contained within the f_{n13} peptide, we examined peptide analogs of f_{n20} with single alanine substitutions in each of the 12 residues of f_{n13} (f_{-2} to f_{10}; subscripts indicate position in sequence relative to alanine) for stimulation of T-cell proliferation in vitro. As shown by the dose-response curves (Fig. 2), several critical substitutions decreased the activity of f_{n20} peptide. A complete listing of Th clones responding to peptide analogs is shown in Table 2. Replacements of Arg-10, Asn-12, and Thr-13 by Ala (f_4, f_2, and f_1) significantly decreased the activity of each clone tested. Replacements of Leu-7 and Thr-8 by Ala (f_7 and f_6) also decreased T-cell activation except for the responses of two clones, BL4L-23 (replacement at f_6) and BP1-3 (replacement

TABLE 1. Th clone proliferation induced by antigenic peptides of various lengths

Peptide	Sequence	ED_{50} (μM) of f_{n20}/ED_{50} (μM) of test peptide for clone:						
		SB14-31 (I)[a]	BL4L-23 (II)	BP2-6 (II)	BP3-7 (III)	BP3-13 (III)	BP1-2 (IV)	BP1-3 (IV)
f_{n20}	DEPLTSLTPRCNTAWNRLKL	1.00 (0.01)[b]	1.00 (0.13)	1.00 (0.31)	1.00 (0.88)	1.00 (1.1)	1.00 (17)	1.00 (17)
f_{n16}	DEPLTSLTPRCNTAWN	0.27	0.15	<0.01	0.81	0.83	0.13	0.04
f_{n13}	LTSLTPRCNTAWN	0.07	0.01	<0.001	0.05	0.05	<0.04	<0.04
f_{n13A7}	AAALTSLTPRCNTAWNAAAA	0.09	0.13	0.04	0.39	0.20	0.12	0.30

[a] Designations in parentheses indicate clone groups.
[b] Values in parentheses are ED_{50}s (μM).

at f_7). This suggests that these five residues are important for T-cell activation. Other single Ala substitutions were still stimulatory for most of the Th clones. These residues may play the role of a spacer, implying that their specific side chain is not crucial for interactions with either the MHC or TCR and that the amino acid must be in this position to allow for the correct conformation. However, there are several exceptions: BP1-2 is unresponsive to Thr-5 and Pro-9 substitutions, BP1-3 is more responsive to Leu-4 and Cys-11 substitutions, and BP2-6 is less responsive to Trp-15 substitutions but more responsive to Cys-11 substitutions.

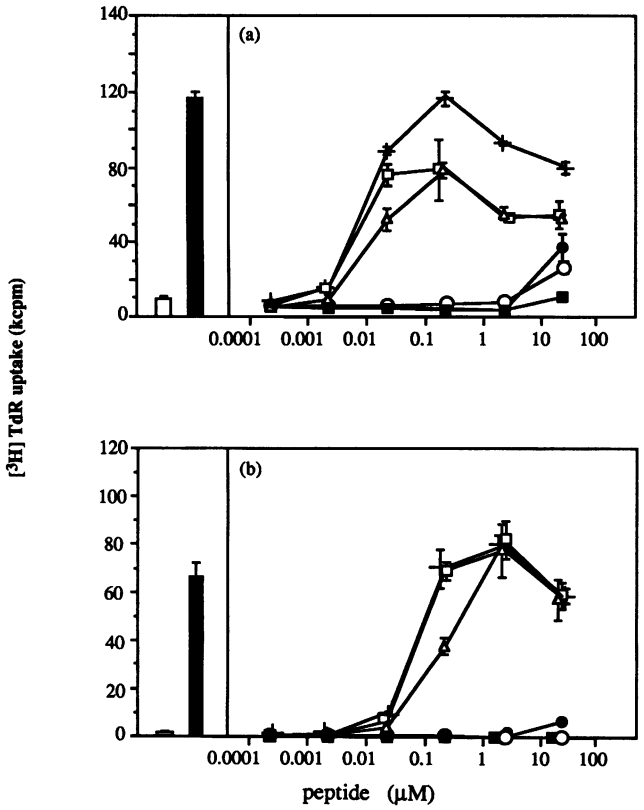

FIG. 2. Proliferation of Th clones SB14-31 (a) and BL4L-23 (b) stimulated by peptides with Ala substitutions. The peptides used were f_{n20} (+), f_1 (○), f_2 (●), f_3 (□), f_4 (■), and f_5 (△). The bars on the left sides of the panels show the proliferative responses of each Th clone after stimulation with Friend virus-induced tumor FT-5 (6) as a positive control (solid bar) and with antigen-presenting cells alone as a negative control (open bar). Error bars show standard deviations. [³H]TdR, [³H]thymidine.

Key MHC and TCR contact residues in peptides. The residue in the position at which the stimulatory activity was lost because of an Ala substitution may be essential for contacting either the MHC or TCR molecule. Such a nonstimulatory peptide can either compete or fail to compete with f_{n20} peptide in a competition assay. We classify the former as the TCR contact residue, because the substituted residue should not be critical for binding to MHC, and we classify the latter as the MHC contact residue, because the substituted residue should be involved in contacting the MHC molecule.

We tested five Ala-substituted peptides (f_1, f_2, f_4, f_6, and f_7) which failed to activate the most sensitive SB14-31 clone (Fig. 3). Three peptide analogs with substitutions at Leu-7, Thr-8, and Asn-12 showed competition for the presentation of f_{n20}. This indicates that these residues are involved directly or indirectly in contacting the TCR. Conversely, two peptide analogs with substitutions at Arg-10 and Thr-13 were unable to compete for the presentation of f_{n20}, suggesting that these are MHC contact sites.

DISCUSSION

Immunization of mice with a syngeneic F-MuLV-induced tumor, FBL-3, elicited a dominant population of cytotoxic T cells, but $CD4^+$ Th cells were elicited at a very low frequency (less than 1 in 500) (6). Moreover, most of the $CD4^+$ Th cells were autoreactive. They proliferated in response to the syngeneic spleen cells without the addition of irradiated FBL-3 cells. In this study, we showed the usefulness of f_{n20} peptide priming in isolating $CD4^+$ Th clones specific to the F-MuLV *env* antigen. Th clones showing a high sensitivity to the antigenic peptide were isolated from a culture containing 0.5 μg of peptide per ml (0.21 μM) after being primed with 10 μg of the peptide. Higher concentrations of synthetic peptides for priming and clonal selection in vitro were ineffective for isolation of Th clones of high sensitivity. An excess of f_{n20} peptide was inhibitory to in vitro proliferation of a highly responsive clone (Fig. 1 and 2). Several boostings after the primary immunization to induce high-titer antibody responses were also ineffective for isolation of $CD4^+$ Th clones, possibly because of negative selection for unknown reasons. Further improvements in peptide immunization and clonal selection may contribute to the generation of better Th responders.

Antigen peptide f_{n20} recognized by two FBL-3-specific Th clones was longer than the 15-residue peptide which resides within the class II binding groove (2). To determine if a portion of the peptide outside the class II binding groove contributes to T-cell activation, we truncated the peptide by several residues from both the amino end and the carboxyl end (f_{n16} and f_{n13}) (Table 1). The effects of decreasing peptide length on T-cell proliferation were distinctive for each clone and were especially drastic for group II and IV Th clones. Group II, III, and

TABLE 2. Relative stimulative activities of peptides with Ala substitutions on proliferation of Th clones

Peptide	Sequence	ED_{50} (μM) of f_{n20}/ED_{50} (μM) of test peptide for clone[a]:						
		SB14-31 (I)	BL4L-23 (II)	BP2-6 (II)	BP3-7 (III)	BP3-13 (III)	BP1-2 (IV)	BP1-3 (IV)
f_{-2}	----------A----	1.0	0.41	0.45	0.67	0.45	0.13	ND[b]
f_{-1}	----------A-----	3.5	0.13	0.08	0.56	0.52	0.10	ND
f_{n20}	DEPLTSLTPRCNTAWNRLKL	1.0	1.0	1.0	1.0	1.0	1.0	1.0
f_1	----------A-------	<0.004	—[c]	—	—	—	—	—
f_2	----------A--------	<0.004	<0.002	—	<0.02	<0.03	—	—
f_3	----------A---------	1.4	1.0	4.9	1.3	1.3	0.35	16
f_4	---------A----------	<0.004	—	—	<0.02	<0.03	—	—
f_5	--------A----------	0.52	0.12	0.11	0.88	0.31	0.04	0.31
f_6	-------A----------	<0.004	0.15	—	—	—	—	—
f_7	------A----------	—	<0.0002	<0.006	—	—	—	7.5
f_8	-----A----------	1.3	1.0	0.87	1.0	0.88	0.89	1.0
f_9	----A----------	0.54	0.24	0.45	1.3	0.72	<0.03	0.12
f_{10}	---A----------	0.86	1.9	1.7	1.7	1.9	2.9	6.6

[a] Designations in parentheses indicate clone groups.
[b] ND, not done.
[c] —, no stimulation of Th with 20 μM peptide.

IV Th clones were less responsive to the shortest peptide, f_{n13}, but their activities were restored with the 20-residue peptide elongated with polyalanines. This T-cell-dependent effect of peptide length strongly suggests that regions of the peptide which lie outside the presumed class II peptide binding groove contribute to the activation of T cells. Earlier studies (12) had also shown that the minimally active antigenic peptide of cytochrome *c* recognized by class II-restricted T-cell hybridomas is 13 residues but that the maximal activity of the peptides is achieved only by the longer 22- to 23-residue peptides. Additional contacts of peptide may be necessary for the stability of the class II structure, as suggested previously (9).

Naturally processed peptide fragments bound to the I-A^b molecule showed a putative consensus sequence of 13 residues (XXNXXXXXPXXXX) in which the conserved residues Asn-3 and Pro-9 are likely to be involved in anchoring the peptide to I-A^b molecules (11). In this study, however, two residues (Arg-10 and Thr-13) of active antigenic peptide f_{n20} bound to I-A^b molecules were identified as MHC contact residues. Since the two closely positioned anchor residues were not found in the consensus sequence, secondary binding pockets of I-A^b molecules may be involved in binding the f_{n20} peptide. The irrelevant peptide of 16 residues of the pigeon cytochrome *c* sequence known to bind to I-A^b (AEGFSYT DANKNKGIT) (10) failed to compete with f_{n20} (unpublished results).

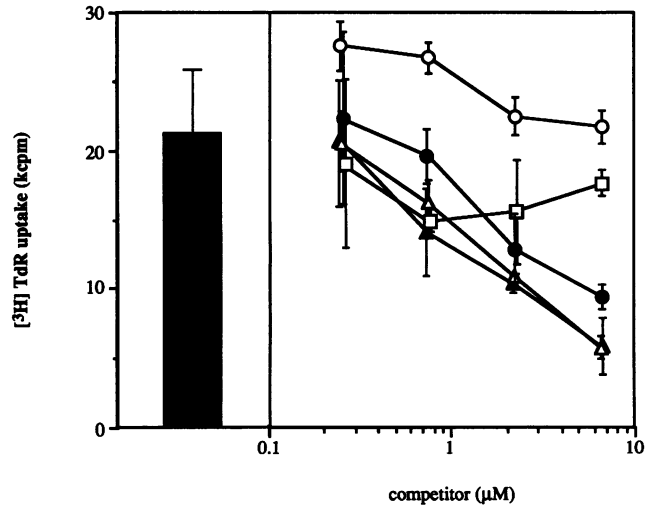

FIG. 3. T-cell proliferation and competition between f_{n20} and peptides with Ala substitutions. The proliferative responses of Th clone SB14-31 stimulated by the f_{n20} peptide (0.08 μM) without competitors (bar) or with competitor peptides f_1 (□), f_2 (●), f_4 (○), f_6 (▲), and f_7 (△) are shown. Peptide f_{n20} and competitor peptides were added simultaneously to the microwell culture. Error bars show standard deviations. [^{3}H]TdR, [^{3}H]thymidine.

Several model systems with T-cell hybridomas have been developed to investigate peptide interactions with MHC class II molecules and with TCRs, including egg white lysozyme (1), myelin basic protein (4), cytochrome *c* (7), and insulin (13). Our findings with a panel of T-cell clones differing in activation responses have identified three residues (Leu-7, Thr-8, and Asn-12) of f_{n20} peptide as the epitope recognized by the I-A^b-restricted Th clones. As exceptions, alanine substitution at Thr-8 resulted in a modest 5-fold decrease in activity for a BL4L-23 clone and alanine substitution at Leu-7 resulted in a 7.5-fold increase in activity for a BP1-3 clone. Other spacer residues (Leu-4, Thr-5, Pro-9, Cys-11, and Trp-15) also interacted with the TCR regions of specified clones (BP1-2, BP1-3, and BP2-6). Thus, the diverse T-cell responses to a single peptide that were observed may reflect greater flexibility in the exposed side chains of an antigenic peptide.

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