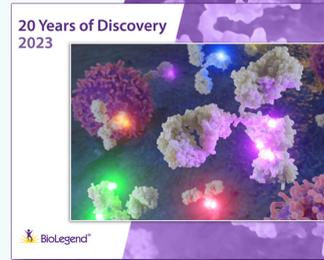


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J Immunol (1999) 162 (12): 7155–7161.

<https://doi.org/10.4049/jimmunol.162.12.7155>

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Identification of Peptide Superagonists for a Self-K-ras-Reactive CD4⁺ T Cell Clone Using Combinatorial Peptide Libraries and Mass Spectrometry¹

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The proliferative responses of a human CD4⁺ T cell clone 29.15.2, reactive with a self-K-ras-derived peptide (³EYKLVVVGAGGVGKSALT²⁰), were tested using a set of X9 combinatorial peptide libraries containing the flanking residues (EYKLVXXXXXXXXXSALT, where X indicates random amino acids). Certain peptide libraries, such as EYKLVXXXXXXXXMXXSALT and EYKLVXXXXXXXXHXSALT, stimulated a marked proliferation of 29.15.2. However, no combinations of substitutions tested, such as EYKLVXXXXXXXXMHXSALT, exhibited additive effects. We subsequently synthesized peptides with degenerate sequences (a mixture of 480 species), where each position is composed of the wild-type (wt) residue or of amino acids that induced the proliferation of 29.15.2, in positional scanning. Interestingly, one fraction of degenerate peptides, separated by reverse-phase HPLC, stimulated much higher proliferation than did the wt; in addition, the retention time of this fraction was distinct from that of the wt. Mass spectrometry analysis of this fraction and flanking fractions identified five peptide species that exhibit strong signals in a manner that parallels the antigenic activity. Finally, 17 candidate peptide sequences were deduced from mass spectrometry and hydrophobicity scoring results, of which two peptides (EYKLVVVGAGGMLKSALT and EYKLVVVGAGGMIKSALT) did induce 52- and 61-fold stronger proliferation, respectively, compared with the wt. These findings indicate that: 1) synthetic peptides that carry “the best” residue substitution at each position of combinatorial peptide libraries do not always exhibit superagonism, and 2) such a drawback can be overcome with the use of mass spectrometry. This approach provides new perspectives for the accurate and efficient identification of peptide superagonists. *The Journal of Immunology*, 1999, 162: 7155–7161.

Although the recognition of peptide Ags by T cells via the TCR has exquisite specificity, many studies have shown flexibility in such recognition (1–3). Recent studies indicated that even certain peptides minimally homologous with a wild-type (wt)³ peptide exhibit stronger proliferation-inducing activity than did a wt on relevant T cells, thereby designated peptide superagonists. From pathogenic and therapeutic points of view, such peptide species should have meaningful implications in autoimmunity, infectious immunity, and cancer immunity (4–6).

Hemmer et al. reported that unbiased approaches using completely randomized combinatorial peptide libraries lead to identification of peptide superagonists for a particular T cell clone (7). In their study, X_n peptides (X₁X₂X₃. . . . X_n; n ≥ 9) induced proliferative responses of a T cell clone that recognizes a myelin basic

protein fragment in the context of DRB1*1501 and in a manner where longer peptides led to stronger responses. Thus, X₁₁ peptide-based combinatorial libraries stimulated proliferative responses sufficient for positional scanning. However, because the clone recognized X₉ sequences as a minimum core, more than two flames were observed in the recognition patterns (7). To avoid such a shift in the recognition patterns and to have sufficiently high responses to assess the recognition patterns for identification of peptide superagonists, we synthesized a set of X₉ combinatorial peptide libraries with flanking residues of the core sequence recognized by a K-ras-reactive CD4⁺ T cell clone. Based on the results for each position of the antigenic peptide, we synthesized several artificial peptides and tested their potential to induce proliferation of the T cell clone. Unexpectedly, none of these peptides induced stronger proliferative responses than did the wt. In the present study, we used mass spectrometry to overcome drawbacks; in addition, we identified two peptide species that exhibit markedly potent stimulation compared with the wt.

Materials and Methods

Peptides

Peptides with defined sequences were synthesized using a solid-phase simultaneous multiple peptide synthesizer, PSSM-8 (Shimadzu, Kyoto, Japan), based on the fluorenylmethoxycarbonyl (Fmoc) strategy and using a 10-fold molar excess of single Fmoc-amino acids; next, the peptides were purified using C18 reverse-phase (RP)-HPLC. In the case of degenerate peptides, the introduction of randomized sequence positions was done in a double coupling step with equimolar mixtures of Fmoc-L-amino acids, used in an equimolar ratio with respect to the coupling sites of the resins (all positions except Cys have 19 aa residues). In the case of combinatorial peptide libraries, the synthesis was performed on a 96-well peptide synthesizer (model SRM96A, Shimadzu) using the same strategy as for degenerate peptides. Degenerate or combinatorial peptide mixtures cleaved

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Received for publication January 25, 1999. Accepted for publication April 6, 1999.

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¹ This work was supported in part by grants-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan, a research grant for intractable diseases from the Ministry of Health and Welfare of Japan, the Ichiro Kanehara Foundation, Ohyama Research Foundation, Japan Rheumatism Foundation, the Pollution-related Health Damage Compensation and Prevention Association, and The Naito Foundation.

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³ Abbreviations used in this paper: wt, wild type; Fmoc, fluorenylmethoxycarbonyl; RP, reverse phase; TFA, trifluoroacetic acid; m/z, mass/charge.

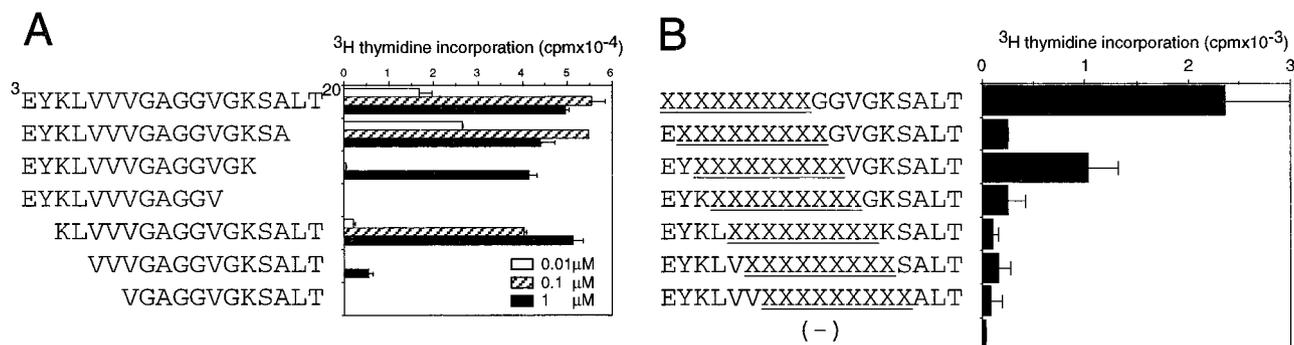


FIGURE 1. Identification of a core sequence of the peptide K-ras p3–20 for recognition by T cell clone 29.15.2. *A*, Various K-ras-derived peptides truncated either from the N terminus or from the C terminus of the self-K-ras-derived peptide p3–20 were tested for their capacity to stimulate T cell clone 29.15.2. T cells were cultured for 72 h in the presence of 0.01 μM (open bar), 0.1 μM (hatched bar), and 1 μM (closed bar) of each truncated soluble peptide and irradiated autologous PBMCs. *B*, Nine consecutive residues on the wt sequence are replaced by random amino acids (designated X). T cells were cultured for 72 h in the presence of 250 μM of each soluble peptide and irradiated autologous PBMCs. Medium control responses without peptide were <200 cpm in all experiments. All data are expressed as the mean value of duplicate determinations \pm SE.

from resins in the presence of 2-methylindole were precipitated and subsequently washed five times with ice-cold anhydrous ethyl ether. Peptide precipitates dried by purging nitrogen gas, dissolved in trifluoroacetic acid (TFA), precipitated with ethyl ether, and dried again. These preparations were dissolved in 50% acetonitrile containing 0.01 N HCl and lyophilized on a Speed Vac (Savant Instruments, Farmingdale, CA). After weight measurement, dried peptide preparations were dissolved in absolute DMSO at 50 mM (based on the assumption that the average molecular mass of amino acid residues is 110); next, preparations were stored in aliquots at -80°C . When used for culture, peptides dissolved in DMSO were diluted to 1 mM in culture medium, centrifuged to remove precipitation, and filtrated for purposes of sterilization. In foregoing studies (8), amino acid composition analysis using X_n peptides solubilized in distilled water (i.e., after removal of precipitates) exhibited the smallest content of Met (3.8%) and the largest content of Asp (11.1%); for other amino acids, it was between 4.0% (Val) and 10.4% (Glu), thus showing an insignificant deviation of amino acid contents. Amino-terminal sequencing analysis of X_n peptides showed insignificant deviation in the molar ratio of residues among the positions sequenced (8).

Assessment of T cell responses

A human CD4⁺ T cell clone recognizing a K-ras-derived peptide in the context of DR51 molecules (DRA + DRB5*0102) was used throughout the study (9). T cells were fed weekly with 50 U/ml human rIL-2 and irradiated autologous PBMCs prepulsed with the wt K-ras p3–20 (EYKLVVVGAGGVGKSALT). The Ag-induced proliferation of the T cell clone was assayed by culturing the T cells (3×10^4 /well) in 96-well, flat-bottom culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ) in the presence of a peptide(s) and irradiated autologous PBMCs (1.5×10^5 /well) using RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 $\mu\text{g}/\text{ml}$ of streptomycin, and 10% heat-inactivated autologous plasma. For the proliferation assay, cells were cultured for 72 h in the presence of 1 mCi/well of [³H]thymidine during the final 16 h.

Separation of peptides on RP-HPLC

The degenerate peptides cleaved from resins were loaded onto a 3.9×150 -mm C18 RP-HPLC column (Waters, Milford, MA) equilibrated with 0.06% TFA in water. The column was eluted at a flow rate of 1.0 ml/min at room temperature in a gradient of increasing acetonitrile concentrations in 0.052% TFA with the A_{214} and A_{280} continuously monitored. Thirty-second (0.5 ml) fractions were collected and lyophilized with a Speed Vac (Savant). The peptide concentration of each fraction was determined based on the assumption that the average molecular mass of a peptide species is 2000.

Hydrophobicity analysis

The relative hydrophobicity scores of the peptides were calculated based on the hydrophobicity scores of each amino acid (10) at relative position numbers 3, 5, 6, 7, 8, and 9 (Ala, +1.8; Glu, -3.4; Gly, -0.4; His, -3.2; Ile, +4.4; Lys, -3.9; Leu, +3.8; Met, +1.9; Pro, -1.5; Ser, -0.8; Thr, -0.6; Trp, -0.9; Tyr, -1.2; and Val, +4.2), because amino acids at relative position numbers 1, 2, and 4 were conserved among peptides. The

relative hydrophobicity score of the wt K-ras p3–20 (EYKLVVVGAGGVGKSALT, where the second Val is relative position 1 and where relative positions 3, 5, 6, 7, 8, and 9 are underlined) is -1.3 ($= -0.4 - 0.4 - 0.4 + 4.2 - 0.4 - 3.9$).

Mass spectrometry

Mass spectra were recorded on a TSQ700 mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a Finnigan electrospray ionization source. The sample was eluted at a flow rate of 3 $\mu\text{l}/\text{min}$ with buffer containing 40% acetonitrile and 0.1% acetic acid mixture in water. For operation, the electrospray voltage was set to 4.5 kV and capillary temperature was 230°C . The sheath gas was set at 40 psi using prepure nitrogen (99.999%). The spectra were acquired by scanning over a 400–2000 mass/charge (m/z) range every 1 s for 3 min. The computer program BIOMASS was used for the integration of every spectrum for scanning time and for calculating the mass in the expected range (11).

Results

Positional scanning by a set of X9 combinatorial peptide libraries containing flanking residues

The CD4⁺ T cell clone 29.15.2 recognizes the self-K-ras-derived peptide p3–20 (³EYKLVVVGAGGVGKSALT²⁰) in the context of DR51 (DRA + DRB5*0102), as described in our previous study (9). To test whether T cell clone 29.15.2 can be scanned with multicombinatorial peptide libraries, we first synthesized X_n -based peptide libraries with various flanking residue sequences (i.e., X17 K, KGX10GK, KGX10GKGGK, KGX9GK, KGX9GKGGK, etc.). A proliferation assay was performed, but none of the libraries induced proliferative responses. We subsequently made sets of a combinatorial peptide library, based on KGX9GK, with each set containing one defined and eight randomized sequence positions on X, against which proliferative responses of 29.15.2 T cells were determined. However, T cells exhibited no response to any set of the library, even at a peptide concentration of 500 μM (data not shown). Because of these negative results, we synthesized a set of X9-based combinatorial peptide libraries that carry flanking sequences, the objective being to design candidate peptide superagonists.

To identify a functional core sequence of the peptide K-ras p3–20 for recognition by 29.15.2, a series of peptides truncated either from the C or N terminus were synthesized, and reactivities of 29.15.2 cells were determined. As shown in Fig. 1A, truncation of the N-terminal ⁷V⁸V or of the C-terminal ¹⁵G¹⁶K completely abrogated the proliferative response of 29.15.2. When nine consecutive residues on the wt sequence were replaced by random amino acids, replacement of ⁷VVVGAGGVG¹⁵, ⁸VVGAG

wild-type: EYKLVVVGAGGVGKSALT
 relative position #: 123456789
 library design: EYKLVXXXXXXXXXSALT

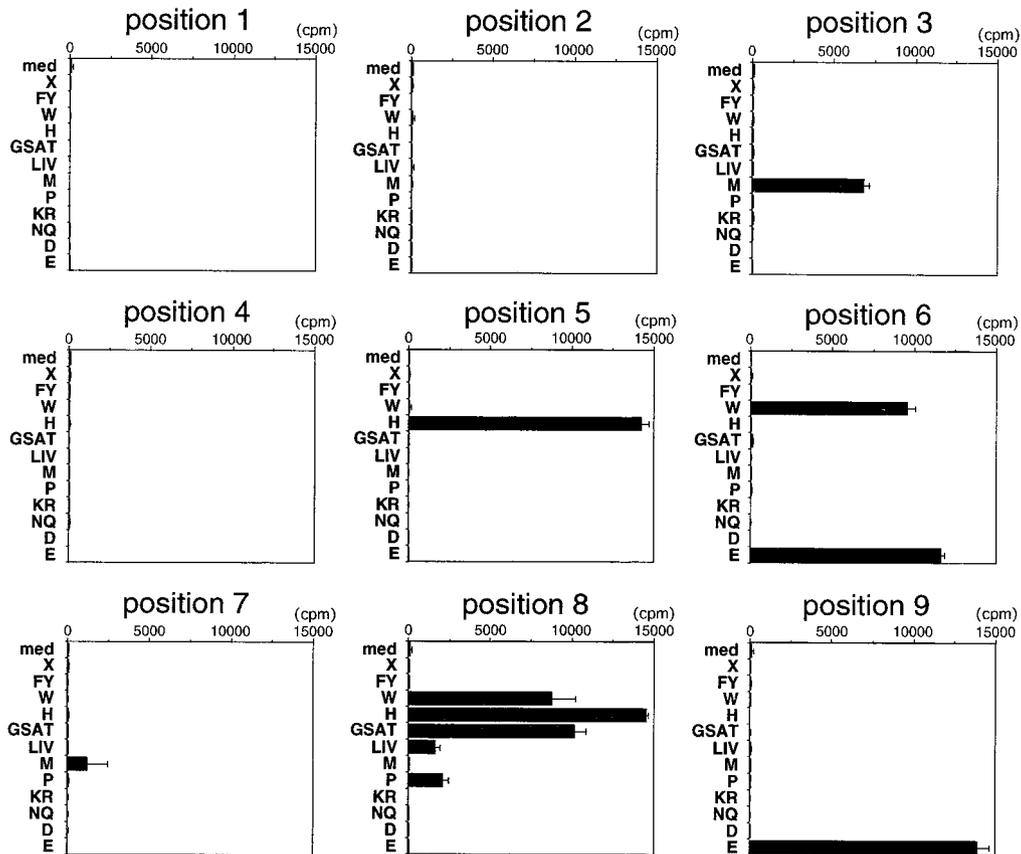


FIGURE 2. Positional scanning by a set of X9 combinatorial peptide libraries containing flanking residues. The relative position number and library design of the self-K-ras-derived peptide p3–20 are shown on the top. For example, a peptide (shown in the upper right panel) (position 3) that stimulated marked proliferation has the sequence of EYKLVXXMXXXXXXXXXSALT. Because amino acids with similar characteristics were combined (FY, GSAT, LIV, KR, and NQ), peptides with W, H, G or S or A or T, L or I or V, and P at position 8 were judged as agonists. Peptides were tested at 150 μ M for proliferative responses of 29.15.2 T cells. Medium control responses without peptide were <200 cpm in all experiments. All data are expressed as the mean value of duplicate determinations \pm SE.

GVGK¹⁶, or ⁹VGAGGVGKS¹⁷ by X9 markedly abrogated the response (Fig. 1B).

Based on these observations, we synthesized a set of X9 combinatorial peptide libraries that carry the flanking residues of the core sequence (i.e., based on EYKLVXXXXXXXXXSALT); we tested their proliferation-inducing activity on 29.15.2 at 150 μ M. As shown in Fig. 2, Met at relative position 3; His at relative position 5; Trp and Glu at relative position 6; Met at relative position 7; Trp, His, Gly, Ser, Ala, Thr, Leu, Ile, Val, and Pro at relative position 8; and Glu at relative position 9 were effective for the induction of proliferation. To identify peptide superagonists based on these results, we subsequently synthesized peptides that carry amino acids positive for proliferation-inducing activity. As shown in Fig. 3, certain peptides, such as Met at relative position 7 and His at relative position 8 on the wt background, did induce stronger proliferative responses than did the wt. However, these single amino acid modifications did not exhibit additive effects when combined in one peptide species (EYKLVVVGAGGMHKSALT; Fig. 3, upper panel, in which residues distinct from the wt are underlined). This was also the case when all of the positions were replaced by “the best” amino acid (EYKLVVVM^AH^EM^HE^S).

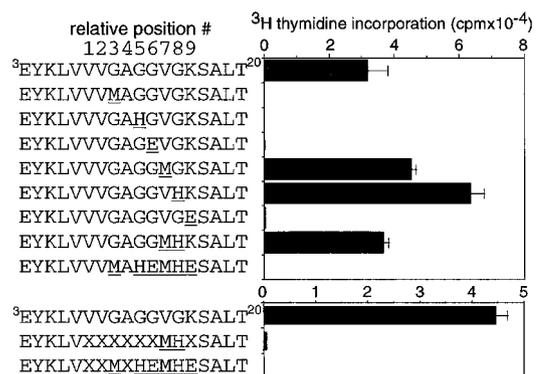


FIGURE 3. Combinations of the best residue substitution at each position do not always result in superagonists. Residues distinct from the wt are underlined. Peptides in the upper panel were tested at 0.1 μ M. Peptides in the lower panel were tested at 100 μ M (wt at 1 μ M). All data are expressed as the mean value of triplicate determinations \pm SE.

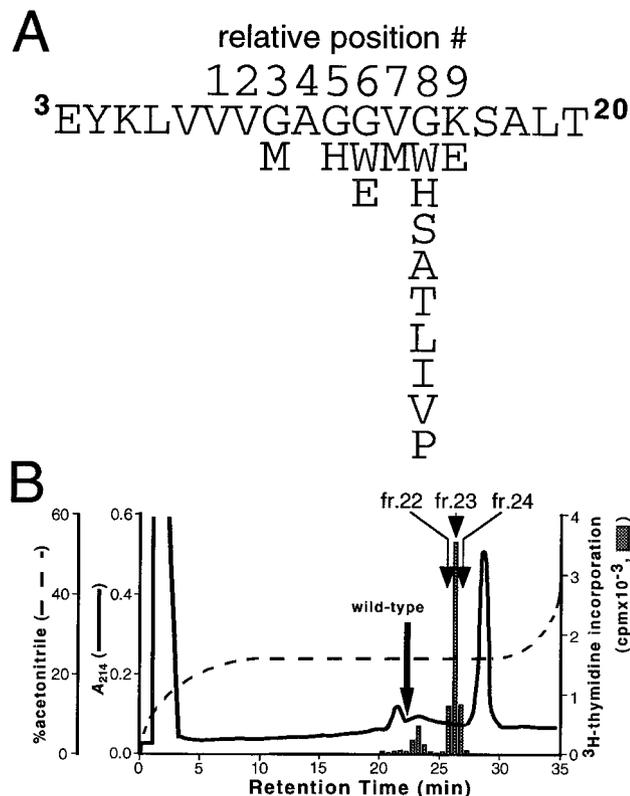


FIGURE 4. Separation of degenerate peptides on RP-HPLC. *A*, The design of degenerate peptides consisting of 480 species ($2 \times 2 \times 3 \times 2 \times 10 \times 2$). For example, relative position 6 is composed of Gly, Trp, and Glu. *B*, Degenerate peptides were separated on C18 RP-HPLC, with A_{214} (solid line) continuously monitored. A dashed line indicates % acetonitrile content. Shaded bars indicate the proliferative responses of 29.15.2 cells at 0.01 μM . The retention time of the wt peptide (bold arrow) was determined on another chromatography (data not shown).

SALT). Moreover, these modifications on the X9 background did not induce proliferative responses even at 100 μM (EYKLVXXX XXXMHXSALT and EYKLVXXMXHEMHESALT; Fig. 3, lower panel). All of these observations indicate that synthetic artificial peptides that consist of combinations of the best residue substitution at each position do not always exhibit superagonism.

Separation of degenerate peptides on RP-HPLC and determination of molecular mass by mass spectrometry

Because the peptide superagonists were not readily identified by combining amino acids that exhibited proliferation-inducing activity in positional scanning, we synthesized a mixture of peptides with degenerate sequences, where each position is composed of the amino acids that induced the proliferative responses of 29.15.2, as shown in Fig. 2. The design of the degenerate peptides depicted in Fig. 4A consists of 480 species ($2 \times 2 \times 3 \times 2 \times 10 \times 2$). For example, relative position 6 is composed of the wt-derived Gly, together with Trp and Glu that exhibited proliferation. Next, we separated the degenerate peptides by RP-HPLC, and the proliferation-inducing activity ($[^3\text{H}]$ thymidine incorporation) of the 30-s fractions was determined (Fig. 4B). Most of these fractions exhibited practically equal levels of proliferative responses at 1 μM (data not shown). However, at a lower peptide concentration, such as 0.01 μM , one fraction stimulated markedly higher proliferative responses than did the wt, the retention time (fraction 23; 26.0–26.5 min) of which was distinct from that of the wt (22.1 min, bold arrow on Fig. 4B), determined on another chromatography (data not shown).

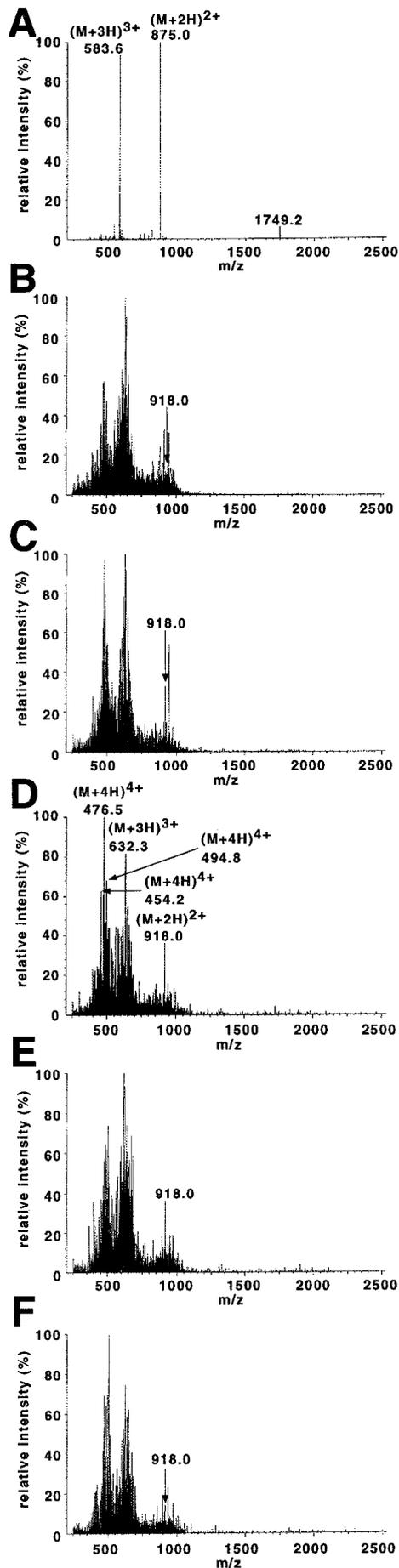
We subsequently analyzed fractions 21–25 and the wt K-ras p3–20 by mass spectrometry. As shown in Fig. 5A, two strong signals of the wt, m/z 583.6 ($[\text{M} + [3\text{H}]]^{3+}$) and 875.0 ($[\text{M} + 2\text{H}]^{2+}$), indicated that the molecular mass was 1748, which corroborates well with the theoretical molecular mass of the wt peptide. As expected, however, fraction 23 exhibited many peaks in the 400–1000 m/z range (Fig. 5D). Theoretically, the peptide superagonist(s) that contributes to the high activity of fraction 23 should: 1) exhibit a higher signal in fraction 23 than in other fractions; and 2) be distinguishable from contaminating small peptides, based on the presence of multivalent ionized forms. By comparing fraction 23 (Fig. 5D) with fractions 21 (Fig. 5B), 22 (Fig. 5C), 24 (Fig. 5E), and 25 (Fig. 5F), we identified five peptide species, 918.0 ($[\text{M} + 2\text{H}]^{2+}$), 476.5 ($[\text{M} + 4\text{H}]^{4+}$), 632.3 ($[\text{M} + [3\text{H}]]^{3+}$), 494.8 ($[\text{M} + 4\text{H}]^{4+}$), and 454.2 ($[\text{M} + 4\text{H}]^{4+}$), that fit the above criteria, because the molecular mass of 480 peptide species should be between 1748.07 (EYKLVVVGAGGVGKSALT) and 2193.62 (EYKLVVVMAGGVGKSALT). Therefore, we expected that some or all of these five signals originated from peptide superagonist molecules.

Hydrophobicity analysis and identification of peptide superagonists

Based on the five candidate signals for peptide superagonists, theoretical molecular masses were calculated (Table I). Next, we listed all of the possible sequences that fall into the range of the theoretical molecular mass $\pm 0.15\%$, the putative error of this system. A total of 5, 9, 9, 11, and 1 sequences (total of 35) were listed, based on signals of m/z 918.0, 476.5, 632.3, 494.8, and 454.2, respectively. We subsequently calculated the relative hydrophobicity scores of these sequences (Table I), as described in *Materials and Methods*. Because the wt peptide was eluted much earlier than fraction 23 on RP-HPLC with the gradient of increasing acetonitrile content (Fig. 4B), it was highly likely that the peptide superagonists contained in fraction 23 were more hydrophobic than the wt. Thus, we selected peptide sequences more hydrophobic than the wt (relative hydrophobicity score being -1.3). All of these peptides (scores ranging from $+3.2$ to $+0.5$) and some peptides slightly more hydrophilic than the wt (scores ranging from -1.5 to -2.4) were synthesized and purified on HPLC; in addition, their activities were tested. As shown in Fig. 6, three peptides induced proliferative responses of 29.15.2 more efficiently than did the wt. The ED_{50} values determined by the dose response curves of the wt (0.11 μM) and all of the peptides tested are summarized in Table I. Thus, two peptides (EYKLVVVGAGGVGKSALT and EYKLVVVGAGGVGKSALT) at much a lower concentration (an ED_{50} of 0.0021 and 0.0018 μM , respectively) than the wt and one peptide (EYKLVVVMAGGVGKSALT) at a similar level of concentration (an ED_{50} of 0.050 μM) induced a stronger proliferation of 29.15.2 T cells than did the wt. As expected, these peptides were apparently more hydrophobic than the wt (scores of $+0.6$, $+1.2$, and $+3.2$, respectively). Thus, among five peptides with scores more hydrophobic than the wt, three exhibited stronger activity than did the wt, thus making for an effective prediction of peptide superagonists.

Discussion

Identification of peptide superagonists was based on use of various strategies. In our previous studies, we identified a peptide superagonist for K-ras-reactive T cells by single amino acid modification on a residue that is critical for binding to class II HLA molecules (6). Thus, only the most N-terminal anchor position (P1) can be replaced by amino acids that interact with HLA-DR molecules with higher affinity, without affecting the recognition by



TCR (5). In this respect, the peptide superagonists identified in our current study were derived from substitutions of relative positions 7 and 8, which are both unlikely to be the P1 anchor position. Thus, these substitutions may affect peptide interactions with TCR or interactions to both HLA and TCR. Indeed, we tested the TCR antagonism of analogue peptides and found that peptide analogues such as ³EYKLVVVVGGAGGPGKSALT²⁰ (where Val¹⁴ is replaced by Pro) function as TCR antagonists against wt-induced responses (data not shown). Thus, it is conceivable that at least one of the substituted residues of the superagonists shown in this study interacts with TCR (5). We subsequently investigated the binding of biotinylated wt and peptide superagonists to L cells transfected with restriction HLA molecules (HLA-DRA + DRB5*0102; Ref. 12) using a FACScan analyzer (Becton Dickinson, Mountain View, CA) (13). However, the binding was below detectable levels, and we could not determine the precise mode of interaction (data not shown).

Simple positional scanning approaches using combinatorial peptide libraries have identified peptide superagonists. Vergelli et al. reported that single amino acid modifications exert additive effects when combined in one peptide species containing multiple substitutions (14). Hemmer et al. reported that unbiased approaches using completely randomized combinatorial peptide libraries led to the identification of peptide superagonists for a particular T cell clone (7), which was also based on the assumption that synthetic artificial peptides that carry the best residue substitution at each position of combinatorial peptide libraries do exhibit additive effects, resulting in superagonists. Another study based on single amino acid substitutions of a wt peptide also exhibited additive effects (15). However, the results shown in our current study are controversial. Thus, synthetic artificial peptides that carry the best residue substitution at each position of combinatorial peptide libraries do not necessarily result in superagonists; rather, “unexpected” amino acids at other positions play important roles in superagonism. However, such an observation may not apply to T cells with a distinct peptide specificity or with distinct HLA restriction patterns; one might assume that there are highly flexible interactions, especially in the case of peptides interacting with TCR or HLA with low affinity. Thus, peptide superagonists might be more readily identified in T cell responses to self-Ags (16) or to cryptic epitopes (17–19).

From this point of view, although replacement by Met at relative position 3 did not induce proliferative responses (EYKLVVVVAGGVGKSALT; Fig. 3), EYKLVVVVAGGVVAKSALT as shown in Fig. 6, induced marked proliferation. Thus, replacement of the wt Gly by Ala at relative position 8 (which is five residues apart from relative position 3), led to T cell activation. Consequently, it seems reasonable to speculate that the best residue substitution at each position does not always mean the best residue, when combined with the best residues at other positions. In this regard, our previous study on a single residue polymorphism at DRβ³⁷, which interacts with the C-terminal p8 or p9 residue of the antigenic peptide accommodated in the peptide-binding grooves of DRB1*0403 and DRB1*0406, indicated that a polymorphism of DRβ³⁷ induced conformational changes of analogue peptides substituted at residues far from a contact site(s) to the polymorphic

FIGURE 5. Determination of molecular mass by mass spectrometry. The synthetic wt K-ras-derived peptide p3–20 (A) was tested using mass spectrometry. Signals of m/z equaling 583.6 and 875.0 correspond to the double- and triple-protonated wt K-ras-derived peptide p3–20, respectively. The ion spectrums of fractions 21 (B), 22 (C), 23 (D), 24 (E), and 25 (F) are shown.

Table I. Peptide sequences, relative hydrophobicity scores, and proliferative responses

Observed Peak (m/z)	Theoretical m.w.	m.w.	Sequence	Relative Hydrophobicity Score ^a	ED ₅₀ ^b		
583.6/875.0 ^c	1748	1748.07	EYKLVVVGAGGVGKSALT	-1.3	0.11		
918.0	1834	1834.15	EYKLVVVGAGEVAKSALT ^d	-2.1	∞ ^e		
		1835.09	EYKLVVVGAGEVVAESALT	-1.6	∞		
		1836.23	EYKLVVVGAGGMLKSALT	+0.6	0.0021		
		1836.23	EYKLVVVGAGGMIKSALT	+1.2	0.0018		
		1836.23	EYKLVVVMAGGVAKSALT	+3.2	0.050		
476.5	1902	1900.22	EYKLVVVGAGEVHKASALT	-7.1	NT ^f		
		1900.22	EYKLVVVGAGEVHGKSALT	-7.1	NT		
		1900.28	EYKLVVVGAGEHGMPSALT	-7.5	NT		
		1901.16	EYKLVVVGAGEVHESALT	-6.6	NT		
		1901.16	EYKLVVVGAGEHVESALT	-6.6	NT		
		1901.22	EYKLVVVGAGEHGMPSALT	-7.0	NT		
		1902.30	EYKLVVVGAGEHGMVSALT	-1.8	∞		
		1902.30	EYKLVVVMAGGVHKSALT	-1.8	∞		
		1902.30	EYKLVVVMAGEHGVGKSALT	-1.8	∞		
		632.3	1894	1892.19	EYKLVVVGAGWVAESALT	+0.9	∞
1892.25	EYKLVVVGAGEMPKSALT			-7.7	NT		
1893.19	EYKLVVVGAGEMPESALT			-7.2	NT		
1894.27	EYKLVVVGAGEMVKSALT			-2.0	∞		
1894.27	EYKLVVVMAGEVGVGKSALT			-2.0	∞		
1894.33	EYKLVVVMAGGMPSALT			-2.4	∞		
1895.21	EYKLVVVGAGEMVESALT			-1.5	∞		
1895.21	EYKLVVVMAGEVGVGKSALT			-1.5	∞		
1895.27	EYKLVVVMAGGMPEASALT			-1.9	∞		
494.8	1975			1973.28	EYKLVVVGAGEHMPESALT	-10.0	NT
				1974.36	EYKLVVVGAGEHMPKSALT	-4.8	NT
		1974.36	EYKLVVVMAGEVHKASALT	-4.8	NT		
		1974.36	EYKLVVVMAGEHGVGKSALT	-4.8	NT		
		1974.42	EYKLVVVMAGEHGMPSALT	-5.2	NT		
		1975.30	EYKLVVVGAGEHMPESALT	-4.3	NT		
		1975.30	EYKLVVVMAGEVHESALT	-4.3	NT		
		1975.30	EYKLVVVMAGEHVESALT	-4.3	NT		
		1975.36	EYKLVVVMAGEHGMPSALT	-4.7	NT		
		1976.33	EYKLVVVGAGEHMTKSALT	-9.6	NT		
		1976.44	EYKLVVVMAGEHGMPSALT	+0.5	∞		
		454.2	1813	1811.09	EYKLVVVGAGGMSASALT	-3.5	NT

^a Hydrophobicity scores were determined as explained in *Materials and Methods*.

^b ED₅₀ values are peptide concentrations (μM) required for 50% of the plateau proliferative responses induced by each peptide.

^c Data on the wt peptide.

^d Amino acid residues distinct from the wt peptide are underlined.

^e ∞, infinity

^f NT, not tested.

DRβ³⁷ (20). These observations indicate that a substitution at a certain residue would induce conformational changes of peptides and affect other residues far from the substituted site. It is thereby conceivable that the same rule applies for the negative activity of peptides EYKLVVVMAGEHMPESALT and EYKLVVVMAGEHGVGKSALT, as shown in Fig. 3. From this viewpoint, the design of the degenerate peptides depicted in Fig. 4A might miss the strongest superagonists. However, the degree of degeneracy (480 species) is reasonable for the current strategy using mass spectrometry, and it would have been practically impossible to perform further detailed analysis if the degree of degeneracy was greater.

In the chromatogram shown in Fig. 4B, there was a major peak at 28.0–29.0 min of retention (fraction 28). However, quantitative amino acid composition analysis of this fraction (data not shown) exhibited only 45% of peptide content compared with fraction 23 (6780 and 14962 pmol for fractions 28 and 23, respectively), which suggests that nonpeptide substances such as the chemicals used for cleavage reaction may contribute to the A₂₁₄ of the 28–29-min peak. This finding also

indicates that the difference in concentrations for each peptide species eluted in distinct fractions was practically negligible in searching for peptide superagonists.

When analyzed using electrospray ionization mass spectrometry, signals from one peptide species are detected as multivalent ionized forms. For example, two strong signals of the wt, m/z 583.6 ([M + [3H]]³⁺) and 875.0 ([M + 2H]²⁺), indicated the molecular mass to be 1748, which corroborates well with the theoretical molecular mass of the wt peptide. This is the merit for electrospray ionization mass spectrometry, compared with time-of-flight mass spectrometry, because the existence of multivalent ionized signals allows successfully synthesized peptides to be distinguished from contaminating small peptide molecules. Indeed, by comparing fraction 23 with fractions 21, 22, 24, and 25, we identified five peptide species that exhibit the highest signal in fraction 23, and these were distinguishable from contaminating small peptides based on the presence of multivalent ionized forms. It is to be noted that we synthesized several artificial peptides based on other higher signals in fraction 23 that did not fit the

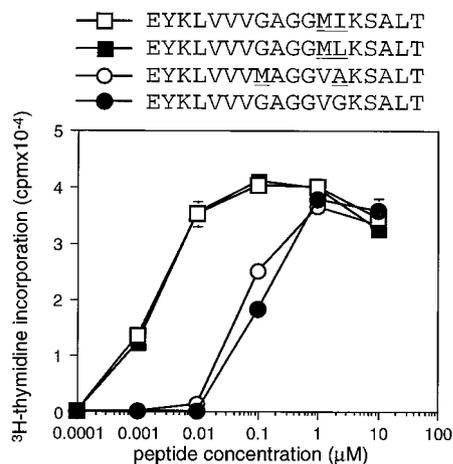


FIGURE 6. Dose response curves of peptide superagonists in comparison with wt K-ras p3–20. T cells were cultured for 72 h with irradiated autologous PBMCs in the presence of each soluble peptide at the indicated concentrations (0.1 nM to 10 μ M). Medium control responses without peptide were <200 cpm. All data are expressed as the mean value of triplicate determinations \pm SE.

above criteria, purified them on HPLC, and tested them for superagonism. Indeed, none of these artificial peptides induced proliferative responses (data not shown).

In this study, combinatorial peptide libraries were synthesized simultaneously using a 96-well synthesizer, SRM96A, to attain uniform quality throughout the positions to be scanned. For this purpose, amino acids with similar characteristics were combined (FY, GSAT, LIV, KR, and NQ). However, even within such groups (for example, Ala and Ser), certain T cell clones, including one reported in our previous study, exhibit agonist/antagonist properties (2, 21). It is conceivable that completely randomized libraries allow for the identification of larger numbers of superagonists.

One set of an X9-based combinatorial peptide library that carries flanking sequences consists of 1.7×10^{10} (19^8) different peptide species, which results in assay concentrations of 8.8×10^{-9} μ M for each single peptide at a final total peptide concentration of 150 μ M. Because the minimum concentration of the superagonist to induce proliferation was 1×10^{-3} μ M, it may be that many different peptide species contribute to stimulation of the T cell clone.

Ras is a protooncogene that encodes p21^{ras}, and point mutations in this gene are involved in carcinogenesis of many different types of malignancies. Mutation of the transforming p21^{ras} gene is limited at codons 12, 13, and 61 (22), two of which are included in the peptide used in the present study. Indeed, human T cells that recognize ras protein and/or peptide have been described previously (6, 23–25); these cells were induced by stimulating a large number of PBMCs or spleen cells with peptides or ras protein in vitro. Certain T cells recognizing a wt ras peptide are capable of recognizing mutated and cancer-associated ras peptides more efficiently (6). These T cells can play a role in host-cancer interactions (25), and adoptive transfer of these T cells may be one strategy for cancer immunotherapy. It is conceivable that the superagonists described herein may be useful for stimulating T cells ex vivo, for purposes of cancer immunotherapy. mAbs that increase efficiency in establishing self-Ag-reactive T cell lines (9) may possibly have additive effects in vitro or ex vivo for the expansion of cancer-reactive T cells from PBMCs.

In conclusion, the current approach with combinatorial peptide libraries and mass spectrometry provides new perspectives for the accurate and efficient identification of peptide superagonists.

Acknowledgments

We thank T. Nakashima (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) for technical assistance and discussion and M. Ohara for comments on the manuscript.

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