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SINGLE AMINO ACID SUBSTITUTION ALTERS T CELL DETERMINANT SELECTION DURING ANTIGEN PROCESSING OF *Staphylococcus aureus* NUCLEASE[1]

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The effect of amino acid residues outside of T cell determinant regions of *Staphylococcus aureus* nuclease (Nase) on the activation of T cell hybridomas has been investigated. T cell hybridomas derived from BALB/c mice immunized with Nase were screened against a nested set of overlapping synthetic peptides spanning the entire Nase molecule. Five regions of Nase, encompassing residues 1 to 20, 21 to 40, 61 to 80, 101 to 120, and 112 to 130, were found to be the T cell determinants. Region 61 to 80 is the immunodominant site. Mutants of Nase with a single amino acid substitution outside the defined T cell determinants were tested for their ability to stimulate the T cell hybridomas. The substitution of arginine for glutamic acid at residue 43 markedly reduces the antigenic potency of the protein for I-E^d restricted T cell hybridomas, which recognize Nase peptides comprised of residues 21 to 40 (p21-40) or 112 to 130 (p112-130). In contrast, the stimulatory capacity of this mutant for I-A^d restricted T cell hybridomas remains unchanged. Our results suggest that selective regulation of an immune response may be achieved by appropriately mutagenizing protein Ag.

Helper T cells generally recognize processed protein Ag in association with class II MHC molecules (Ia) (1). Although the mechanisms of Ag processing remain elucidated, it has been demonstrated that protein fragments and synthetic peptides, but not native proteins, can stimulate T cells when presented by metabolically blocked APC, suggesting that Ag are degraded before being presented to T cells (2–4). These synthetic peptides are believed to mimic the end-products of Ag processing, and they bind to the single binding site of MHC molecules (5–9).

A protein Ag usually has multiple potential T cell determinants that can be demonstrated if synthetic peptides containing the sequence of the determinants are used for immunization (10, 11). However, T cells preferentially respond only to certain of the determinants if

native proteins are used as immunogens. Accumulating evidence suggests that Ag processing plays a critical role in determining the repertoire of immunogenic peptides available for interaction with Ia molecules and subsequent recognition by T cells (12–16).

The approach that we have taken to understand how processing events generate immunogenic peptides is to substitute single amino acids outside of defined T cell epitopes and determine their effect on T cell determinant selection. In this report, we have analyzed the immune response in BALB/c mice to Nase,[3] a bacterially derived protein composed of 149 amino acids (17). We provide evidence that a single amino acid substitution outside of two defined I-E^d restricted T cell determinants can greatly influence the immune response toward both determinants.

MATERIALS AND METHODS

Animals. BALB/c mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

Ag. Peptides were prepared as described previously (18), based on the Nase sequence determined by Shortle (17). The mutagenized (at residues 26, 43, 60, 85, 113, and 115) Nases were prepared by the two-primer oligodeoxynucleotide-directed site-specific mutagenesis procedure of Zoller and Smith (19) (E. Uhlmann, and J. A. Smith, unpublished observations). The bacterial expression and isolation of Nase and R43 (i.e., a mutagenized form of Nase in which arginine is substituted for glutamic acid at residue 43) were carried out as described by Serpersu et al. (20) using the expression plasmid pFOG405 and *Escherichia coli* strain SE6004. The concentrations of the stock solutions of Ag were determined by amino acid analysis using a Beckman 6300 amino acid analyzer.

Culture medium. All cell cultures and assays were performed in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 5×10^{-5} M 2-ME.

Antibodies. mAb against mouse L3T4 (anti-CD4) and mouse Lyt-2 (anti-CD8) were purchased from Becton Dickinson. The antibodies were dialyzed against RPMI 1640 medium before assays.

Cell lines. BW5147.G.4.Oau^R.1 was from Dr. R. Schwartz. A20.2J(I-A^d, I-E^d) was from Dr. J. Kappler and Dr. P. Marrack. TA3(I-A^{d/k}, I-E^{d/k}) was from Dr. L. Glimcher. Class II MHC L cell transfectants RT 2.3.3H (I-A^d) and RT 10.3H2 (I-E^d) were from Dr. R. Germain. CTLL.2 was from Dr. D. Raulet.

Isolation and characterization of T cell hybridomas. Mice were immunized with 100 μg of Nase and R43 in CFA. Seven days later, popliteal, para-aortic, and inguinal lymph nodes were removed and pressed through a fine wire mesh to prepare a single cell suspension. The cells were cultured at 4×10^6/ml with 17 μg/ml Nase or R43 in a T-25 cm² flask with a upright position for another 3 days. Viable cells were then isolated by fractionation with lympholite M and fused with BW5147 cells, as described previously (21). When hybridomas appeared, their MHC restriction and fine specificity were determined by screening them against a nested set of overlapping synthetic peptides spanning the entire Nase molecule using class II MHC L cell transfectants, expressing either A^d or E^d molecules, as APC. All the

[3] Abbreviations used in this paper: Nase, *Staphylococcus aureus* nuclease; R43, a mutagenized form of Nase with arginine substituted for glutamic acid at residue 43.

T cell hybridomas reported herein were subcloned at least once by limiting dilution method.

IL-2 assay. Cells (1 × 10^5) of a T cell hybridoma, 5 × 10^4 cells of an APC (A20.2J, TA3, or L cell transfectants), and various concentrations of Ag in a final volume of 0.25 ml were plated in duplicate or triplicate in microtiter plates. At 18 to 24 h later, 100 µl of supernatant were removed and frozen for more than 2 h at −70°C. IL-2-dependent CTLL.2 cells (1 × 10^4) were added to warmed supernatants. After 24 h in culture, 0.5 µCi of [³H]thymidine was added per well. At 6 h later, the cells were harvested by a semi-automated cell harvester (Skatron, Inc., Sterling, VA), and thymidine incorporation was measured by scintillation counting.

Lymphocyte proliferation assay. Lymphocytes were prepared from lymph nodes of mice primed with Ag (0.012 µmol of Nase or Nase peptides), as described above. The lymphocytes were plated in microtiter plates at 4 × 10^5 cells/well with Ag in total volume of 0.2 ml. For antibody inhibition studies, 0.2 µg of anti-CD4 or 1 µg of anti-CD8 antibodies was added to each well at the initiation of the lymphocyte proliferation culture. At 48 h later, 0.5 µCi of [³H]thymidine was added to each well. The cells were harvested 18 to 24 h later, and thymidine incorporation was measured.

RESULTS

T cell hybridomas derived from BALB/c mice immunized with Nase were screened against a nested set of two 19-residue peptides and 12 20-residue peptides covering the whole Nase molecule. Five regions, 1 to 20, 21 to 40, 61 to 80, 101 to 120, and 112 to 130, were identified as T cell determinants (Table I). The MHC restriction of the Nase-specific T cell hybridomas was determined using class II MHC L cell transfectants, expressing either I-A^d or I-E^d molecules, as APC. The T cell hybridomas responding to p1-20, p61-80, and p101-120 were I-A^d restricted, and those responding to p21-40 and p112-130 were I-E^d restricted. The T cell hybridomas recognizing these peptides were designated AD1, AD61, and AD101, and ED21 and ED112, respectively.

Of the five T cell determinants, the region 61 to 80 was the immunodominant site. Approximately 85% of the T cell hybridomas were I-A^d restricted and in turn about 85% of those reacted with p61-80. Furthermore, T lymphocytes from BALB/c mice primed with Nase responded to p61-80 and Nase in a lymphocyte proliferation assay. However, the same population of lymphocytes did not proliferate when stimulated with the other four antigenic

peptides. To test whether the low response to these peptides was due to the small size of their corresponding T cell repertoires, mice were immunized with each of the 14 overlapping Nase peptides. The T lymphocytes, primed in vivo with the five antigenic peptides corresponding to the five Nase T cell epitopes, proliferated when stimulated in vitro with the peptide used for the immunization (Table II). In each case, the proliferation of the lymphocytes could be suppressed by addition of anti-CD4 but not anti-CD8 mAb, indicating that the population of lymphocytes responding to the peptides were T helper cells. In contrast, no proliferation was observed using the other nine peptides (i.e., p11-30, p31-50, p41-60, p51-70, p71-90, p81-100, p91-110, p121-140, and p131-149) (data not shown).

It was suggested by Sette et al. (22) that an I-E^d antigenic site could be identified by a "basic-basic-non-charged-basic" motif, and in the case of p112-130, there are the corresponding residues: 121(His), 124(His), 125(Leu), and 126(Arg). However, when truncated forms of p112-130 were used to stimulate ED112, p112-125 lacking an arginine residue at residue 126 was still able to activate ED112 (Fig. 1); p112-125 could also competitively inhibit the activation of ED21 by p21-40 (data not shown). Without residue 126, residue 112 becomes critical for binding to E^d molecule since p113-125 failed to inhibit the activation of ED21. Residue 112 is not likely to be involved in the interaction with the T cell receptor, since p113-126 can still stimulate ED112 (Fig. 1). It is suggested that residue 112 replaces the role of residue 126 in binding to E^d molecules.

Nase mutants with a single amino acid substitution (at residue 26, 43, 60, 85, 113, and 115) were assayed for their ability to be presented to Nase-specific T cell hybridomas. Only the substitution of arginine for glutamic

TABLE I
Distribution of Nase-specific T cell hybridomas with respect to their MHC restriction and fine specificity[a]

	Immunized with	Nase-specific hybridomas	MHC restriction	Fine specificity	
		Number of T Cell Hybridomas			
Fusion 1	Nase	33	A^d 29/33	—	
			E^d 4/33	21–40	1/4
				112–130	2/4
Fusion 2	Nase	51	A^d 49/51	—	
			E^d 2/51	21–40	1/2
				112–130	1/2
Fusion 3	Nase	97	A^d 84/97	61–80	71/84
				1–20	12/84
				101–120	1/84
			E^d 13/97	21–40	10/13
				112–130	3/13
Fusion 4	Nase	70	A^d 60/70	61–80	50/60
				1–20	10/60
			E^d 10/70	21–40	9/10
				112–130	1/10
Fusion 5	R43	62	A^d 62/62	—	
			E^d 0		
Fusion 6	R43	35	A^d 35/35	—	
			E^d 0		

[a] T cell hybridomas were derived from BALB/c mice immunized with Nase or R43. Their MHC restriction and fine specificity were determined as described in *Materials and Methods.*
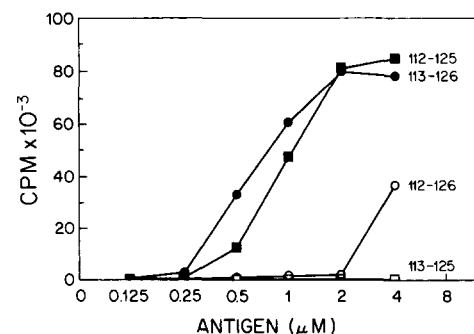
TABLE II
Responsiveness of BALB/c mice to Nase peptides[a]

Immunized with	cpm × 10^{-3} (±SEM)			
	Medium	Peptide	Peptide and anti-CD4	Peptide and anti-CD8
1–20	3.6 (0.2)	13.8 (0.5)	3.2 (0.6)	15.3 (1.4)
21–40	4.7 (0.7)	15.7 (1.6)	3.2 (0.3)	15.9 (1.0)
61–80	5.9 (0.5)	34.2 (3.6)	4.0 (0.4)	31.2 (0.8)
101–120	5.1 (0.5)	13.1 (1.5)	3.7 (0.5)	15.5 (0.4)
112–130	4.5 (0.5)	15.1 (2.0)	3.4 (0.3)	12.5 (1.4)

[a] Lymphocytes were isolated from BALB/c mice immunized with each of the five Nase peptides and incubated with the same peptides used for immunization in the absence or presence of anti-mouse CD4 or CD8 antibodies, as described in *Materials and Methods.*

Figure 1. Reactivity pattern of I-E^d restricted T cell hybridoma, ED112. ED112 cells were incubated with p112-125 (■), p113-126 (●), p112-126 (○), or p113-125 (□) in the presence of A20 cells and assayed for IL-2 production, as described in *Materials and Methods.* Each *data point* is the arithmetic mean of duplicate experiments.

acid at residue 43 markedly reduced that protein's ability to stimulate the I-E^d restricted T cell hybridomas ED112 and ED21 (Fig. 2, A and B). However, the antigenic potency of R43 for the T cell hybridomas AD61, AD1, and AD101 remained unchanged compared with Nase (Fig. 2, C-E). Similar results were obtained using different preparations of R43. To achieve a comparable half-maximal response for the I-E^d restricted T cell hybridomas with A20 as APC, a 20- to 50-fold higher concentration of R43 is needed in comparison with the wild-type Nase (data not shown).
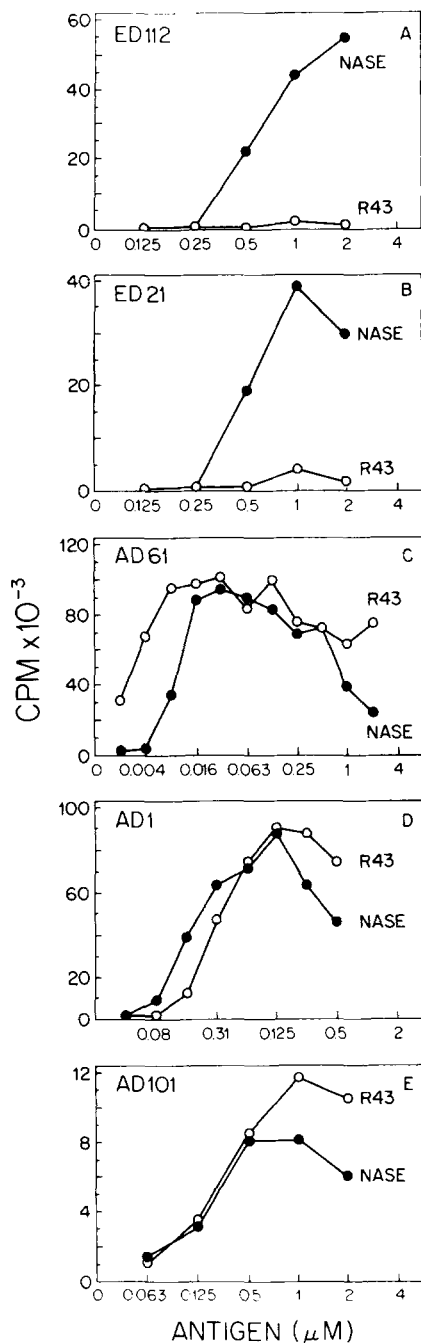
When R43 was used to immunize BALB/c mice, no I-

E^d restricted T cell hybridomas recognizing either p21-40 or p112-130 were obtained from two fusions of six mice (Table I). In contrast, when Nase was used as immunogen, I-E^d restricted T cell hybridomas constituted around 13% of the total Nase-specific T cell hybridomas responding to either p21-40 or p112-130.

We have recently derived from A/J mice a Nase-specific T cell hybridoma, EK112, which like ED112 recognizes the Nase peptide p112-130 (Z. Liu, unpublished observations). To test the possibility that the low response of ED112 to R43 may be due to the reduced production of this fragment during Ag processing, R43 was used to stimulate EK112 when using TA3 (I-A$^{d/k}$ and I-E$^{d/k}$) cells as APC. The dose responses to Nase of ED112 and EK112 were comparable (Fig. 3A). In contrast, the response of ED112 to R43 was much lower than the response to EK112 (Fig. 3B). However, when the fine specificity of EK112 was compared with that of ED112 differences were found between the two T cell hybridomas (Fig. 4). The ED112 but not EK112 could respond to p112-125 and p113-126 (Fig. 4A) and EK112 but not ED112 could recognize p114-127 (Fig. 4B). Although both cell lines responded to p113-127, EK112 is more responsive to the peptide than ED112 (Fig. 4B).

A comparison was made between L cells expressing only E^d molecules and A20 cells expressing both E^d and A^d molecules, as APC. It has been shown that class II MHC density can affect T cell activation (23), and to exclude this possibility, the number of APC used in the assays was adjusted such that A20 cells presented p21-40 to ED21 or p112-130 to ED112 equal to or better than E^d L cells. As shown in Figure 5, E^d L cells present Nase more efficiently to ED21 and ED112 than do A20 cells. For example, as little as 0.063 μM Nase activates ED21 (Fig. 5A) and ED112 (Fig. 5B) when E^d L cells were used. However, 0.5 μM Nase is required to activate the same I-

ANTIGEN (μM)

Figure 2. Response of Nase-specific T cell hybridomas to Nase and R43. Nase-specific T cell hybridomas ED112 (A), ED21 (B), AD61 (C), AD1 (D), and AD101 (E) were cultured with either Nase (●) or R43 (○) with A20 cells. Assays were performed as described in *Materials and Methods.* Each *data point* is the arithmetic mean of triplicate experiments.
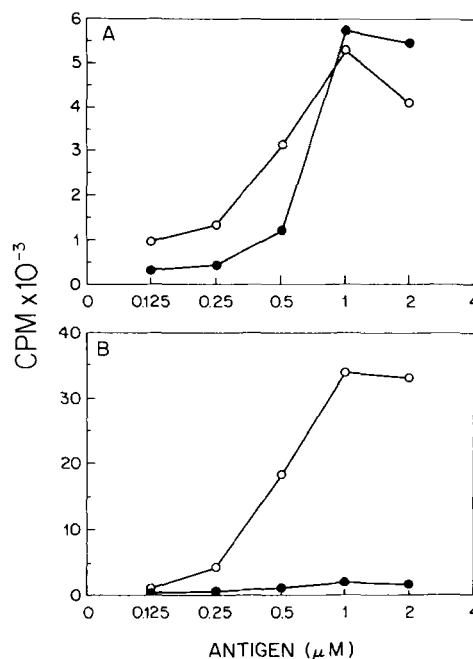

ANTIGEN (μM)

Figure 3. Responses to Nase and R43 of T cell hybridomas with different MHC restriction recognizing the same Nase peptide, residues 112 to 130. ED112 (●) and EK112 (○) were assayed with Nase (A) or R43 (B) with TA3 cells, as described in *Materials and Methods.* Each *data point* is the arithmetic mean of triplicate experiments.
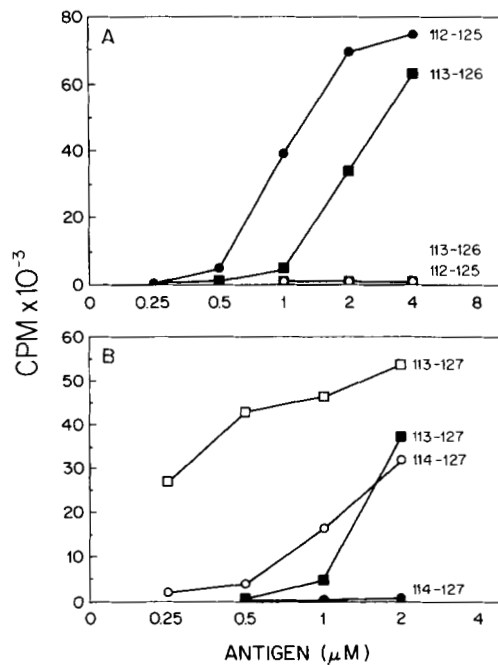
Figure 4. Differences in fine specificities between ED112 and EK112. ED112 (●, ■) and EK112 (○, □) were assayed with p113-126 (■, □) or p112-125 (●, ○) (A); p113-127 (■, □) or p114-127 (●, □) (B), as described in *Materials and Methods*. Each *data point* is the arithmetic mean of duplicate experiments.
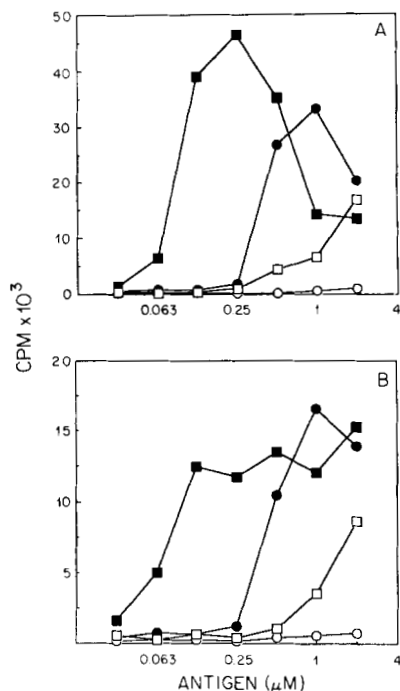

Figure 5. Different antigenic potency of Nase and R43 when presented to T cell hybridoma ED112 by E^d L cells or A20 cells. ED21 cells (A) and ED112 (B) were cultured with either E^d L cells (0.5 × 10^5/well, ■, □) or A20 cells (1 × 10^5/well, ●, ○) in the presence of Nase (■, ●) or R43 (□, ○), as described in *Materials and Methods*. Each *data point* is the arithmetic mean of triplicate experiments.

E^d restricted T cell hybridoma, when A20 cells were used as APC. The antigenic potency of R43 for the I-E^d restricted T cell hybridomas was also tested with E^d L cells as APC. For R43, no E^d-restricted determinants are generated by A20 cells with concentrations of Ag up to 10 μM (data not shown). In contrast, with E^d L cells as the APC, a response by ED21 and ED112 is observed at R43

concentrations of 1 μM and above. Thus, E^d cells are more effective than A20 cells in presenting fragments capable of being recognized by the I-E^d restricted T cell hybridomas (Fig. 5).

DISCUSSION

It is thought that Ag processing plays an important role in determining the ensemble of immunogenic peptides available for interaction with MHC molecules (10–13). After an Ag is taken up by an APC, intracellular processing events lead to either the survival or the destruction of certain regions of a protein Ag. Before investigating how this process works at the molecular level, we have localized the T cell determinants of a model protein, Nase.

Five regions of the Nase molecule, encompassing residues 1 to 20, 61 to 80, 101 to 120, 21 to 40, and 112 to 130, are found to be T cell determinants in BALB/c mice. T cell hybridomas derived from mice primed with Nase recognize the first three regions in association with I-A^d molecules, and the last two regions in association with I-E^d molecules. Sette et al. (22) found that six (p1-20, p11-30, p61-80, p81-100, p91-110, and p101-120) of the 14 overlapping Nase peptides could bind to I-A^d molecules, using an Ia-peptide binding assay. However, our results indicate that of these six peptides only three (p1-20, p61-80, and p101-120) are able to induce an immune response in BALB/c mice. These differences can be explained by a "hole in the T cell repertoire" (24).

One of the two I-E^d-restricted T cell determinants identified by us (residues 21 to 40) is not within the group of seven Nase peptides, which can bind to I-E^d molecules (i.e., p31-50, p51-70, p101-120, p112-130, and p121-140), contain the I-E^d motif (i.e., p1-20, p31-50, p41-60, p112-130, and p121-140) or both (i.e., p31-50, p112-130, and p121-140) (22). Approximately 8% of the Nase-specific T cell hybridomas obtained from mice immunized with Nase respond to p21-40 in the context of I-E^d (Table I); p21-40 can also activate T helper cells from mice immunized with p21-40 (Table II). Since Sette et al. (22) used an in vitro Ia-peptide binding assay, p21-40 may not be able to bind to I-E^d molecules under the conditions used for their assay, and this could account for the difference between our results and theirs. Such disparities emphasize the need for detailed in vivo studies to identify unequivocally T cell determinants.

In the other I-E^d T cell determinant, residues 112 to 130, there is an I-E^d binding motif as described by Sette et al. (22), which is histidine (residue 121), histidine (residue 124), leucine (residue 125), and arginine (residue 126). Using various truncated forms of p112-130, we have shown that residue 126 is not required for binding to E^d molecules, and its role in binding can be subserved by residue 112. This demonstrates that flanking residues in an antigenic peptide may play as important a role in binding to Ia as the motif itself. It is interesting to note that p112-126 is less stimulatory for ED112 than p112-125 and p113-126 (Fig. 1), although p112-126 is more competitive for binding to I-E^d molecules than are the other two peptides (data not shown), suggesting that the presence of both end residues (i.e., residues 112 and 126) interferes with the interaction between the T cell receptor and peptide-Ia complex. This result is further proof that the residues flanking an antigenic site can markedly affect the antigenic potency presumably by altering pep-

tide structure (13, 14).

Of the five T cell determinants of Nase (I-A^d or I-E^d restricted), the region mimicked by p61-80 is identified as the most immunogenic. Among the three I-A^d restricted determinants, it was reported previously that p61-80 did not contain the I-A^d binding motif and that the peptide bound to I-A^d molecules with weaker affinity compared with the other two I-A^d restricted peptides, p1-20 and p101-120, which contained the binding motif (23, 25). This suggests that although binding of peptide to Ia molecules is a prerequisite for mounting an immune response, there appears to be no correlation in this case between the affinity of the peptide for the Ia molecule and its antigenicity. Other factors may account for the difference in the antigenicity of the peptides, such as a limitation of the repertoire of T cells capable of responding to those peptides. For example, Guillet et al. (26) showed that a λ-repressor cI peptide bound to E^d molecules with high affinity, but the peptide was unable to induce an I-E^d restricted immune response in mice, and they attributed this to a "hole in the repertoire." However, such a suggestion does not suffice for the low response of lymphocytes to the other four Nase peptides when Nase is used as immunogen, since each individually can induce T cell proliferation when used as immunogens (Table II). It seems likely that Ag processing also plays an important role in determining the set of available immunogenic sites. Thus, processing events may lead predominantly to the survival of the immunodominant region (i.e., for Nase in BALB/c mice the processed fragment(s) containing residues 61 to 80). One approach that we have taken to understand better the role of Ag processing is to attempt to modulate the selection of epitopes by substituting a single amino acid residue outside defined T cell determinants.

The substitution in Nase of arginine for glutamic acid at residue 43 markedly reduces that protein's ability to stimulate the T cell hybridomas ED21 and ED112 (Fig. 2, A and B). In contrast, the antigenic potency of R43 for the T cell hybridomas responding to p1-20, p61-80, and p101-120 remains unaltered (Fig. 2, C-E). The immunogenicity of R43 was also tested, and no I-E^d restricted T cell hybridomas were obtained from two fusions of six mice primed in vivo with R43. This result suggests that the immunogenicity of the two I-E^d restricted regions of Nase is markedly reduced by this substitution.

It has been shown that residues distant from or close to a T cell determinant can affect the immune response to antigenic sites (13-15, 27). Shastri et al. (15) found that the reactivity differences of two closely related Ag, lysozyme from hen egg white (HEL) and ring-tailed pheasant (REL), to HEL-specific T cell hybridomas was due solely to changes in amino acid residues distant from the T cell determinant. Because there are several amino acid differences between the HEL and REL, the precise substitutions responsible for the effect have not yet been identified.

In our study, a single defined substitution at residue 43 of Nase selectively reduces the antigenicity of two disparate E^d antigenic sites. The substitution at residue 43 did not affect the recognition of the I-A^d restricted determinants, suggesting that the apparent change in processing is not due to aberrant Ag uptake but rather to a change in some intracellular processing event intrinsic to the APC.

One explanation for these results would be that the fragments from R43 containing the E^d-restricted determinants are poorly generated by the APC. We have derived from A/J mice immunized with Nase a T cell hybridoma, which also recognizes p112-130 but in the context of E^k. If the production of fragments containing 112-130 from R43 is reduced, the response to R43 of EK112 should have also decreased. The two T cell hybridomas were tested with Nase and R43 using as APC, TA3 cells (expressing both I-A$^{d/k}$ and I-E$^{d/k}$). Although unable to activate ED112, R43 can still stimulate EK112, and, at the same time, both T cell hybridomas have similar sensitivities for Nase (Fig. 3). This result suggests that the fragments covering 112 to 130 from R43 are not reduced. However, the possibility still exists that the production of the fragments from R43, which ED112 can recognize or is most sensitive to, is reduced, whereas the production of the peptides, which EK112 can respond to, remains unchanged, since further analysis of the fine specificity of the two T cell hybridomas has revealed that ED112 but not EK112 can respond to p112-125, and EK112 but not ED112 can react with p114-127, whereas both hybridomas respond to p113-127 (Fig. 4). Our results and those of others (13, 14, 28, 29) have demonstrated that amino acids flanking T cell determinants can interfere with Ag binding to Ia molecules or bound Ag interaction with the T cell receptor. It is possible that the mutation at residue 43 may influence Ag processing in such a way that the fragments containing residues 21-40 and 112 to 130 from R43 may differ in size and boundary from the end-products from Nase. The flanking residues of the fragments from R43 may interfere with the interaction among the peptides, T cell receptor, and E^d molecules, but not E^k molecules.

Apple and co-workers (11) have proposed that Ag are cleaved after they bind to the MHC molecules. They have shown that a determinant of chicken lysozyme containing residues 21 to 35 is immunodominant in I-A^d expressing mice but that it is not immunodominant in I-A^d/E^d expressing mice, in which an I-E^d restricted determinant encompassing residues 108 to 120 is immunodominant (30). This suggests that preferential contact of class II MHC molecules with certain regions of an Ag prevents the interaction between other regions of that Ag and other class II MHC molecules, and thereby controls subsequent processing. In agreement with the in vivo study of Cogswell and co-workers (30), our in vitro results indicate that E^d L cells present Nase and R43 to ED21 (Fig. 5A) and ED112 (Fig. 5B) more effectively than do A20 cells, implicating a competitive relationship for binding to Ia molecules between E^d and A^d sites in Nase undergoing processing. These results could also be explained by differences in the processing efficiencies between E^d L cells and A20 cells, since it has been reported that even genetically identical Ag presenting cell clones display heterogeneity in Ag processing (16).

We have demonstrated that a single amino acid substitution distant from an antigenic region can markedly influence its antigenic potency. If additional chemical and physical data validate our observations, we predict that rational construction of appropriately mutagenized protein Ag could lead to selective regulation of a desired immune response.

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