Brief Definitive Report

A SINGLE AMINO ACID MUTATION IN A PROTEIN ANTIGEN ABROGATES PRESENTATION OF CERTAIN T CELL DETERMINANTS

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The TCR on $CD4^+$ T cells generally recognizes fragments of protein antigens that are processed by APCs and presented bound to syngeneic class II MHC molecules (1–3). Since most T cells recognize synthetic peptides on the surface of fixed or inactivated APCs, it is presumed that after internalization, intact proteins are processed to a form analogous to that after partial proteolysis (4, 5). The molecular events involved in antigen processing are not clearly understood. We have used recombinant mutant proteins of staphylococcal nuclease that differ from the native protein by one amino acid to investigate the role of structural alterations in protein molecules that affect processing and presentation of protein antigens. Evidence is presented that a single amino acid change in the native protein affects the structure of the processed peptide in such a manner that stimulatory determinants are no longer presented to certain T cell clones, despite the fact that (*a*) other nuclease-specific T cells remain responsive to the mutant protein and (*b*) a synthetic peptide corresponding to the immunodominant region of the mutant protein is highly stimulatory to both groups of nuclease-specific T cells.

Materials and Methods

Mice. B10.A, B10.A (5R), and (B10.A × B10)F_1 male mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

Antigens. Recombinant wild-type staphylococcal nuclease (Nase) and mutant nucleases were prepared from an alkaline phosphatase–staphylococcal nuclease gene hybrid carried on the pFoG405 plasmid generously provided by Dr. David Shortle (6). Proteins were isolated from *Escherichia coli* transformants and purified on CM-Sephadex, C-25 (Pharmacia Fine Chemicals, Piscataway, NJ). Recombinant nucleases were >95% pure by SDS-PAGE and staining with Coomassie blue. Protein concentration was determined by the method of Lowry. Nase peptides were synthesized on a 430a automated peptide synthesizer (Applied Biosystems, Inc., Foster City, CA). Peptides were cleaved from the resin using anhydrous HF and purified by reverse-phase chromatography. Peptides were >95% pure. Peptides were generously provided by Judy Regan and Dr. Kenneth Seamon.

T Cell Clones. Nase-specific T cell clones were derived from immunization of (B6 × A/J)F_1 mice (7). T cells were grown in the presence of antigen 20 U/ml human rIL-2 (Cetus

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Corp., Emeryville, CA), and irradiated (4,000 rad) syngeneic spleen cells in RPMI 1640 containing 7% FCS, 10 mM Hepes, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μM 2-ME, and antibiotics and maintained in culture by restimulation every 2 wk.

Proliferation. T cell activation was measured by incubating 10^4 T cells with 4×10^5 irradiated spleen cells (4,000 rad) with the indicated amount of antigen. The amount of [^{3}H]TdR (specific activity, 1 μCi/well; ICN Radiochemicals, Irvine, CA) incorporated into proliferating cells after a 12-h pulse was assessed at 72 h. The results are expressed as the mean of three replicate cultures. SD was <15%. For reasons of simplicity SD values are not detailed in the data given. All experiments were performed at least three times.

Results and Discussion

We have previously reported that in the response of (B6 × A/J)F_1 mice to the protein staphylococcal nuclease (Nase), E^k-restricted T cell clones preferentially recognize the Nase (81–100) peptide (7). Furthermore, truncation to the 15 amino acid peptide 86–100 preserves maximal T cell stimulation (Finnegan, A., manuscript submitted for publication). One clone, N40, recognizes Nase and the 86–100 peptide in association with both B10.A ($E^k_\alpha E^k_\beta$) and B10.A (5R) ($E^k_\alpha E^b_\beta$) spleen cells used as APC, although 10^2–10^3-fold greater antigen concentration was required to give equal stimulation with $E^k_\alpha E^b_\beta$ APCs (Fig. 1 *A*). When substitutions in the 86–100 peptide were analyzed for T cell stimulatory capacity, all of the E^k-restricted clones recognized the 86–100 peptide substituted at residue 89 (leu to phe) presented by B10.A stimulator cells. However, the potency of the 89-substituted peptide was 10-fold less than the native 86–100 peptide. In contrast, using B10.A (5R) stimulator cells, clone N40 recognized the 89 substituted peptide 10-fold more efficiently than the native 86–100 peptide (Fig. 1 *B*). A change in the class II molecule $E^k_\alpha E^k_\beta$ versus $E^k_\alpha E^b_\beta$ could significantly alter the conformation of the peptide bound to the class II molecule or the orientation of the peptide in the binding site of the class II molecule. These effects could contribute to an increase in the affinity of N40's TCR for the ligand, determined by the 89-substituted peptide and the $E^k_\alpha E^b_\beta$ molecule.

The response of T cells to small peptides may not predict completely the responses to larger proteins containing these peptide sequences. Antigen processing and pre-

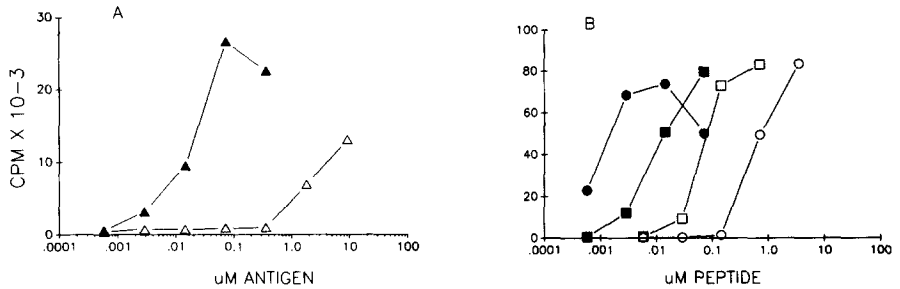

FIGURE 1. (*A*) The T cell clone N40 recognizes Nase more efficiently on B10.A APC ($E^k_\alpha E^k_\beta$) than on B10.A (5R) ($E^k_\alpha E^k_\beta$) stimulator cells. 10^4 clone N40 T cells were incubated with titrated amounts of Nase in the presence of 4×10^5 B10.A (▲) and B10.A (5R) (△) stimulator cells. (*B*) The T cell clone N40 recognizes the 89 substituted (leu to phe) peptide more efficiently on B10.A (5R) ($E^k_\alpha E^b_\beta$) APC than on B10.A ($E^k_\alpha E^k_\beta$) APC. 10^4 clone N40 T cell were incubated with titrated amounts of peptide in the presence of 4×10^5 B10.A, 86–100 peptide (●), 89 substituted peptide (■) and B10.A (5R), 86–100 peptide (○), 89 substituted peptide (□).

sentation may be influenced by changes in amino acid sequences that do not have equivalent effects on T cell responses to small synthetic peptides. To test this possibility T cell responses to a series of recombinant staphylococcal nuclease mutant proteins with single amino acid changes were examined. These mutant nucleases were constructed by gap misrepair mutagenesis of the staphylococcal nuclease gene and isolated from *E. coli* transformants (6). The mutant protein with an amino acid change at residue 89 (leu to phe) was analyzed since this corresponds to the substitution in the synthetic 86-100 peptide that did not abrogate T cell recognition. Although clone N40 was able to recognize the 89 substituted peptide and the native nuclease (Fig. 1), the response to the corresponding 89 mutant protein was $>10^3$ less potent in the presence of B10.A APC (Fig. 2 *A*). To determine if a processed peptide can be generated from the 89 mutant protein and presented by the $E^k_\alpha E^k_\beta$ molecule, another E^k-restricted, Nase-specific T cell clone was used. Clone G53 recognizes an epitope on the 86-100 peptide different from that recognized by clone N40 (data not shown). In contrast to N40, clone G53 efficiently recognized both the native nuclease and the 89 mutant protein when presented by B10.A stimulator cells (Fig. 2 B). Thus, the B10.A APCs present a processed peptide of the 89 mutant that binds to the $E^k_\alpha E^k_\beta$ class II molecule and is efficiently presented to the clone G53. However, the structure of the endogenously produced peptide derived from the 89 mutant protein must be different from that derived from the native nuclease protein since binding of the processed peptide to the $E^k_\alpha E^k_\beta$ molecule efficiently presents the epitope recognized by clone G53 but not the epitope recognized by clone N40.

Next, the ability of B10.A (5R) APC to process and present the 89-mutant protein to clone N40 was tested since clone N40 recognizes the synthetic peptide substituted

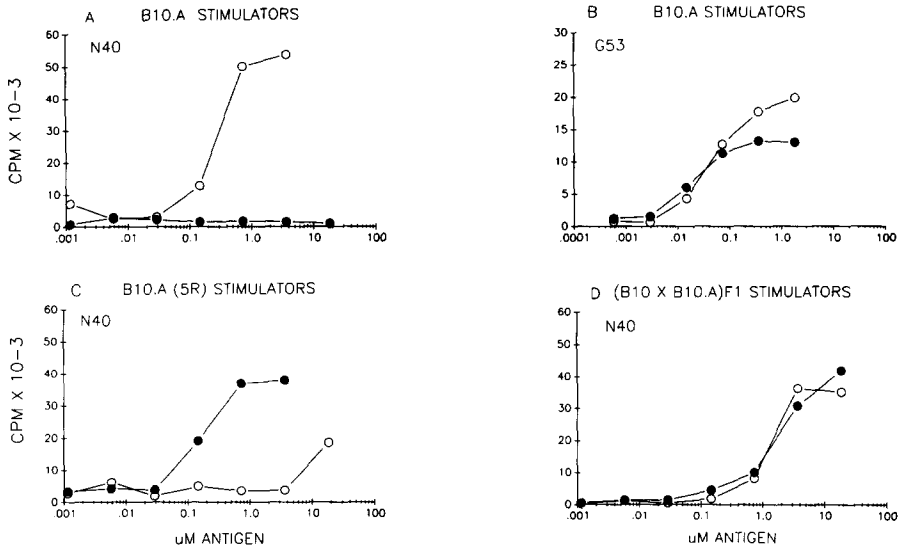

FIGURE 2. B10.A APC can present the 89 mutant nuclease to clone G53 but not clone N40, whereas B10.A (5R) and (B10 × B10.A)F_1 APC can present the T cell epitope recognized by clone N40. Clone N40 stimulated with B10.A (*A*), clone G53 stimulated with B10.A (*B*), clone N40 stimulated with B10.A (5R) (*C*), and (B10 × B10.A)F_1 (*D*), in the presence of wild-type Nase (○) and in the presence of 89 mutant nuclease (●).

at 89 more efficiently with B10.A (5R) APC than with B10.A APC. Interestingly, N40 effectively recognized the 89 mutant protein when processed and presented by B10.A (5R) E^k_α E^b_β or (B10 × B10.A)F_1 stimulator cells (Fig. 2, *C* and *D*). Furthermore, the 89 mutant protein was recognized more efficiently than the native nuclease protein on B10.A (5R) APC.

A single substitution in the native nuclease protein dramatically affects the fine specificity of T cell recognition. The 89 substitution is located within the region of the molecule recognized by Nase-specific T cell clones but appears to have an effect distal to this region. This is determined by the fact that clone N40 responds to the 89 substituted peptide but not to the 89 mutant protein suggesting that a peptide that is probably larger than 15 amino acids is necessary for the 89 substitution to have its influence. This finding is distinct and complements recent evidence demonstrating that structural differences in homologous proteins that are located outside of the T cell determinant can affect cloned T cell responses. Flanking sequences outside of the minimal peptide required for T cell recognition have been identified that have both positive and negative effects on T cell recognition (8–11). Thus, naturally processed peptides may contain structures that interfere with presentation of the determinants recognized in the synthetic peptide (12, 13). Differences in native and mutant processed peptides could be created (*a*) by changing the location of proteolytic cleavage sites in the mutant protein, (*b*) by the 89 substitution itself influencing the conformation of the processed peptide, or (*c*) by a combination of these mechanisms. Class I- and class II-restricted T cells have been identified that when primed to a particular peptide are unable to respond to the native protein from which the peptide was derived, implying that antigen processing influences which peptides are available for T cell recognition (12, 14, 15).

The change in potency of the mutant 89 protein could be due to a change in affinity of the processed peptide for the class II molecule such that only T cell clones with receptors of high affinity would be triggered by this peptide–class II complex. Clone N40 is in fact a higher affinity clone than the other Nase-specific T cells by the criterion that it requires a lower concentration of antigen to give maximal stimulation (data not shown). It is more likely that the conformation of the peptide when bound to the E^k_α E^k_β molecule results in the failure to express the determinants recognized by clone N40. These determinants could either be specific T cell contact sites on the peptide or sites formed from the interaction of peptide with the class II molecule.

There is a MHC difference in recognition of the processed 89 mutant protein by clone N40. This is unlikely due to genetically determined differences in antigen processing since the background genes of B10.A and B10.A (5R) are identical and would be expected to share non-MHC genes involved in processing. Thus, the MHC difference probably involves the interaction of the processed peptide with allele-specific residues in the class II molecules. A similar result was observed with MHC class I-restricted T cell clones (13). The B10.A and B10.A (5R) E α chains are identical and the E β chains differ at only four amino acids (16, 17). The change in amino acid 89 from leu to phe, which increases the potency of the 86–100 peptide and the mutant protein for presentation by B10.A (5R) APC, may for example help to orient the processed peptides binding to the E^k_α E^b_β molecule in a configuration that efficiently exposes T cell epitopes for recognition. Alternatively, the physical association of the peptides with the E^k_α E^b_β molecule may create a complex that interacts

with the TCR of N40 with high affinity. At present there is no consensus as to the conformation that peptides adopt when bound to class II or that class II molecules adopt when bound by peptides (18, 19).

The evidence presented here implies that antigen processing and/or presentation of single amino acid variants has the potential to influence the fine specificity of T cell recognition. The demonstration that the antigen processing system handles mutant proteins differently from synthetic peptides may be relevant to the design of synthetic peptide vaccines that may not induce an immunological crossreactive response to native proteins.

Summary

Nase-specific T cell recognize the 86–100 peptide in association with B10.A APC. Clone N40 recognizes the 86–100 peptide in association with B10.A (E^k_α E^k_β) and B10.A (5R) (E^k_α E^b_β) APCs. We demonstrate here that a single amino acid substitution in the staphylococcal nuclease protein alters the structure of the processed peptide such that the T cell epitope recognized by clone N40 was only available for recognition in conjunction with B10.A (5R) but not the B10.A APCs. Other Nase-specific T cells recognize the mutant nuclease, and a synthetic peptide corresponding to the immunodominant region of the mutant protein was stimulatory for all the Nase-specific T cells. These results suggest that the mutation either affects the processing of the protein into antigenic peptides or affects the conformation of the processed fragment differently from that of the peptide.

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