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Cutting Edge: Introduction of an Endopeptidase Cleavage Motif into a Determinant Flanking Region of Hen Egg Lysozyme Results in Enhanced T Cell Determinant Display[1]

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Cutting Edge: Introduction of an Endopeptidase Cleavage Motif into a Determinant Flanking Region of Hen Egg Lysozyme Results in Enhanced T Cell Determinant Display[1]

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The choice of which determinants of a whole Ag will be presented on cell surface MHC class II molecules after uptake and processing by APC is the result of the interplay between structural characteristics of the Ag and the processing machinery of the APC. In this study, we demonstrate that introduction of a dibasic motif adjacent to a subdominant determinant enhances the presentation of this determinant from the whole molecule. This is the first report showing that a single amino acid substitution in a whole Ag, designed to introduce an endopeptidase recognition site, enhances display of class II-restricted determinants, most likely by creating a peptide chain cleavage in the antigenic molecule. Our findings have important implications for the understanding of immunodominance and for vaccine design. ***The Journal of Immunology,*** **2000, 165: 20–23.**

Major histocompatibility complex class II-restricted determinants are generally derived from uptake of exogenous Ags by APC. In the endocytic pathway, the Ag is subjected to proteolytic cleavage, which may require unfolding and/or the reduction of disulfide bonds. Proteases that have been implicated in the processing of exogenous Ags include cathepsins B, D, E, L, and S (1, 2) and the recently described asparaginyl endopeptidase (3). The fate of a particular region of a protein Ag is impacted by several factors inherent in the antigenic molecule: 1) the ease of unfolding/reduction, potentially affecting availability for proteolytic cleavage, 2) the localization of endopeptidase cleavage sites on the antigenic molecule, and 3) MHC binding affinities of different areas of the molecule. However, which determinants will ultimately be displayed on the APC surface is determined by the interplay of these properties with the relevant components of the processing machinery of the APC: 1) choice of proteases expressed and their localization, 2) pH in the relevant compartments, affecting proteolytic activity, 3) the particular binding properties of the MHC allele and the intracellular localization of MHC molecules, and 4) impact of chaperones, i.e., DM, DO, and invariant chain.

The impact of residues of the Ag outside the determinant region on MHC class II-restricted presentation has been demonstrated in studies using species lysozyme variants (4) or random single amino acid substitutions in staphylococcal nuclease (5, 6). The results of those studies were interpreted to indicate an effect of the substitutions on Ag processing.

In this study, we addressed the effect of a specific cleavage motif on Ag processing and presentation. We reasoned that the introduction of an endopeptidase cleavage site adjacent to a determinant that is not efficiently generated from the whole protein would result in proteolytic cleavage at this site, thus rendering the determinant more available for binding to the MHC molecule and subsequent presentation on the surface of the APC. This would lead to more efficient stimulation of T cells specific for this particular determinant from the altered as compared with the wild-type molecule. To test this hypothesis, we used the model Ag hen egg lysozyme (HEL).[4] The endopeptidase cleavage motif we chose to introduce was a pair of basic amino acids, one of the well-characterized recognition motifs of the broad family of proprotein convertases (7, 8). The members of this family of endopeptidases specifically cleave carboxy terminally of a pair of basic amino acids. This endopeptidic cleavage is frequently followed by carboxypeptidase-mediated removal of both basic amino acids (9). We show that processing by APC of a mutant HEL containing a dibasic motif results in more efficient presentation of an adjacent MHC class II-restricted determinant than does processing of wild-type HEL.

Materials and Methods

Ag presentation assays

HEL-specific T cell hybridomas used in this study were 6F7 and 1–7B8 (20–35/A^d); 930B2 (11–25/A^d); Ad71 (71–85/A^d); and G28.C9 (106–116/E^d)

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[4] Abbreviation used in this paper: HEL, hen egg lysozyme.

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(10). Ag presentation assays were performed by coculturing 5×10^4 T hybridoma cells and 2.5×10^4 B lymphoma cells (LB27.4 or A20) or 5×10^5 splenocytes with Ag for 20 h. Ag-specific stimulation of the T hybridomas was determined by assaying for IL-2/IL-4 production using the IL-2/IL-4-dependent HT-2 cell line. Proliferation or survival of HT-2 cells was determined either by incorporation of [^{3}H]thymidine (Fig. 1*A*) or by metabolizing MTT and measurement of the OD at 570 nm, respectively. The data are represented as Δcpm and ΔOD, respectively, and were calculated by subtracting the values obtained with medium alone from those obtained with Ag.

Production of recombinant HEL variants

Oligonucleotide-directed mutagenesis of HEL cDNA in pUC18 (a gift from I. Kumagai, Sendai, Japan) was performed according to the instructions provided by the Clontech Transformer Kit from Clontech Laboratories (Palo Alto, CA). Mutations were confirmed by DNA sequence analysis. Mutated HEL cDNA fragments were subcloned into the expression plasmid pMN12 (provided by Dr. T. Blair, Xoma, St. Monica, CA) and transformed into *Saccharomyces cerevisiae* KHK-1 (Leu-2, Ura-3) using the lithium acetate procedure. The complete, mature HEL protein was secreted into the culture medium. The recombinant protein was purified using cation exchange chromatography on CM-Sepharose Fast Flow (Pharmacia, Piscataway, NJ) and gave a single band on SDS-PAGE gels and a single species of the correct m.w. by mass spectrometry (performed by Dr. C. Schroeter, Tübingen, Germany). The mutants had full enzymatic activity as lysozymes (data not shown). Recombinant wild-type HEL purified from *S. cerevisiae* had equivalent stimulatory capacity for specific T cell hybridomas to commercial HEL (Sigma), repurified in our lab as described (Ref. 4 and data not shown). Therefore, wild-type HEL purified from egg whites was used in all experiments described here.

Results

To study the impact of Ag processing on determinant display we chose to analyze a MHC class II-restricted subdominant determinant of HEL in H-2^d mice. In the H-2^d haplotype, the response to HEL is directed toward one dominant determinant (106–116/Ed) and several subdominant (11–25/A^d, 20–35/A^d) and cryptic (71–85/A^d, 1–16/E^d) determinants (10, 11).

T cell hybridomas specific for 20–35, although being quite sensitive for the synthetic peptide, required significantly more HEL for equivalent stimulation. Thus, we postulated that the determinant 20–35, as recognized by these T cell hybridomas, was not efficiently generated by APC processing of whole HEL. Therefore, this subdominant determinant was a good candidate to test our hypothesis that the display of a determinant could be altered by introducing an endopeptidase recognition site adjacent to it. Because residue 33 of HEL is lysine (K), we sought to create a dibasic motif in the whole protein by changing residue 34 from phenylalanine (F) to arginine (R). Endopeptidase cleavage after a pair of basic amino acids is frequently followed by carboxypeptidase-mediated removal of both basic amino acids (9) (in this case K33R34). Accordingly, we predicted that the mixture of peptides displayed on cell surface class II molecules after processing of the mutated HEL by APC would contain peptides not extending beyond residue 32. Therefore, we first determined the core of the determinant for two T cell hybridomas that recognized 20–35/A^d.

The core of the subdominant determinant is 23–32

To determine the boundaries of the core of the determinant, we stimulated the T cell hybridomas with 12-mer peptides overlapping by 11 aa spanning the region from 19–35. As shown in Fig. 1*A*, stimulation is lost when the amino terminus does not include residue 23 (23–34 is still stimulatory) and when residue 32 is lost (21–32 is stimulatory). This identifies the core of the determinant as 23–32.

Because we predicted that residues 33 and 34 would be lost during processing of the mutant HEL molecule, we tested a set of peptides with varying amino termini (*n*) and a fixed carboxy terminus at position 32 (where *n* assumes all residues from 18 to 23; Fig. 1*B*). Only the two shortest peptides, including 23–32, which represents the actual core, had a slightly lower stimulatory capacity

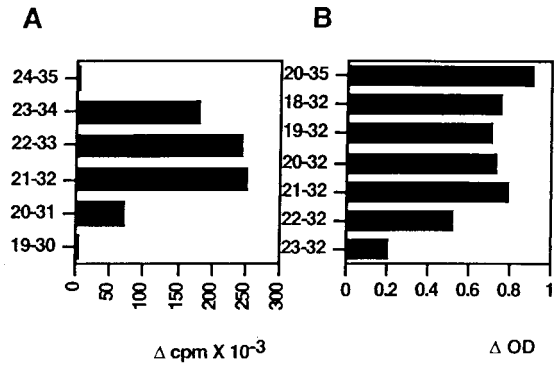

FIGURE 1. The core of the subdominant HEL determinant 20–35/A^d is 23–32. The stimulatory capacities of sets of synthetic peptides for 20–35/A^d-specific T cell hybridomas were tested. *A*, IL-2 response of the T hybridoma 6F7 to 3.5 μM pin peptides presented by B10.GD (A^{d}E^0) splenocytes. *B*, IL-2 response of the T hybridoma 6F7 to 1.2 μM peptides presented by B lymphoma LB27.4 (A^{b,d} E^{b,d}). Similar results were obtained with T hybridoma 1–7B8 as well as when fixed APC were used.

than the determinant envelope 20–35. Importantly, even this core peptide was more stimulatory than native HEL (data not shown and compare Fig. 1*B* (1.2 μM peptide) with Fig. 2*B*).

Nevertheless, it has been shown that residues outside the determinant core can impact T cell stimulation (12). Although the series of peptides displayed in Figs. 1, *A* and *B*, indicate that position 34 is not critical for T cell recognition, we wished to confirm that changing F34 to R would have no effect. As indicated in Fig. 2*A*, peptides 20–35 and 20–35:F34R have equivalent stimulatory capacities for the T cell hybridomas. The same result was obtained with peptides 21–35 vs 21–35:F34K (data not shown). These findings together indicated that the determinant 23–32 was suitable to test our hypothesis by changing F34 to R in the HEL molecule.

Introduction of an endopeptidase cleavage site flanking the determinant 23–32/A^d enhances its presentation

To compare the efficiency of processing of wild-type HEL protein with that of recombinant F34R, each protein was provided to B lymphomas for processing, and the resulting display of specific

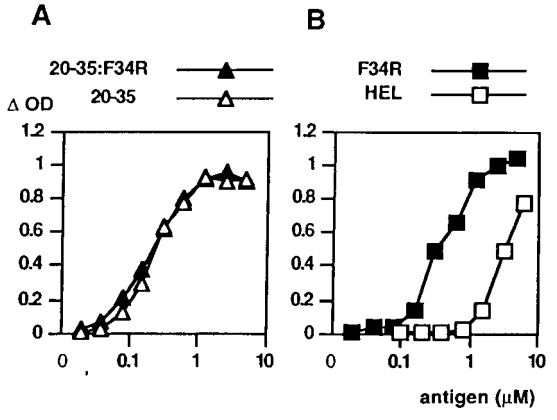

FIGURE 2. More efficient presentation of the determinant 23–32/A^d from the HEL variant F34R containing an endopeptidase cleavage motif. The B lymphoma LB27.4 was cocultured with the indicated concentrations of synthetic peptides (*A*) or native protein (*B*), and the resulting presentation of the determinant 23–32/A^d was determined by the stimulation of IL-2 production from the T hybridoma 6F7. Similar results were obtained using T hybridoma 1–7B8 or using A20 as APC.

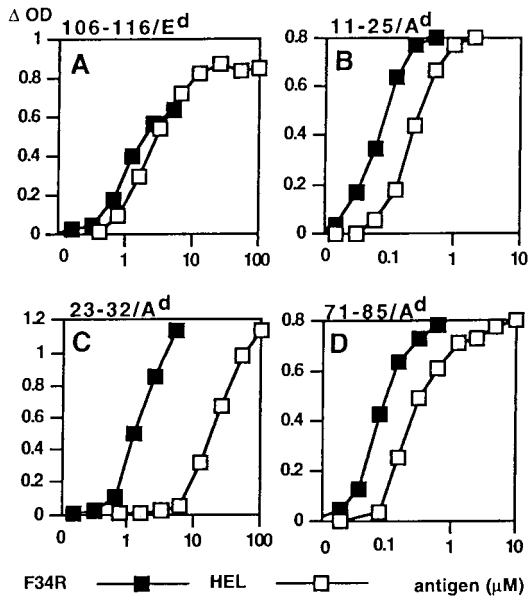

FIGURE 3. Distant determinants of HEL are affected to varying degrees. Dose-response curves from cocultures of LB27.4 APC with HEL or F34R were performed with T cell hybridomas specific for four different determinants: *A*, 106–116/E^d (G28.C9); *B*, 11–25/A^d (930B2); *C*, 23–32/A^d (1–7B8); *D*, 71–85/A^d (Ad71). This is one representative of four independent experiments in which stimulation of all four T hybridomas was assessed in parallel. Using A20 as APC gave identical results.

MHC/peptide complexes on the APC surface was measured as the stimulatory capacity for a 23–32/A ^{d}-specific T cell hybridoma. As represented in Fig. 2*B*, a 9-fold lower concentration of F34R compared with wild-type HEL was required to achieve equal stimulation of the T cell hybridoma 6F7 by the B lymphoma LB27.4. Similar results were obtained with other B lymphomas (A20, M12; data not shown). In over 30 experiments (data not shown), the concentration of HEL required for equivalent stimulation of the T cell hybridomas 6F7 and 1–7B8 was on average 11-fold higher than that of F34R, ranging from 5- to 25-fold. These results indicate that there is a step in the processing of HEL that is affected by the single amino acid exchange (F34R), which introduces a dibasic motif into the molecule.

Distant determinants of HEL are affected to varying degrees

We next analyzed whether introduction of the dibasic motif also affected the processing and presentation of distant determinants of HEL in the same haplotype. Fig. 3*A* shows that HEL and F34R are of comparable efficiency in the stimulation of T hybridoma G28.C9, which is specific for the dominant determinant 106–116/E^d. The presentation of the determinants 11–25/A^d (Fig. 3*B*) and 71–85/A^d (Fig. 3*D*) is 3-fold more efficient from F34R than from HEL. This is in agreement with the notion that altering one processing intermediate by introducing a cleavage site can potentially affect subsequent proteolytic steps by providing an altered substrate. However, the effect is smaller than that for the targeted determinant, 23–32/A^d (Figs. 3*C* and 2*B*). The cryptic determinant 1–15/E^d, which is not generated from whole HEL by splenocytes or B lymphomas (10), was also not presented when F34R was provided (data not shown). Thus, introducing a dibasic motif adjacent to an inefficiently displayed determinant enhanced the presentation of this determinant specifically.

To further corroborate our conclusion that the localization of the dibasic motif is crucial to its effect on the processing of individual determinants, we tested two recombinant HEL mutants in which a dibasic motif had been introduced at position 61/62 (residue 61 is R and residue 62 was changed from W to K or R to give the mutant proteins W62K and W62R, respectively). The T cell hybridoma 3A9 (13) is specific for HEL 46–61/A^k and requires residue 61 for its stimulation (14). In agreement with the notion that both basic residues (here 61/62) are lost upon cleavage at a dibasic motif, stimulation of 3A9 by W62K or W62R was reduced 45- or 15-fold, respectively, compared with wild-type HEL (H. Deng, unpublished results). As shown in Table I, the stimulatory capacity of W62K and W62R was equivalent to that of wild-type HEL for the T cell hybridomas specific for the H-2^d-restricted determinants 23–32, 11–25, and 71–85 and was slightly enhanced for 106–116. Taken together, the results of this study indicate that a dibasic motif can affect the processing of HEL and can alter the display of a specific determinant, dependent upon its localization in the molecule.

Table I. *Ratio of EC_{50} for presentation of HEL determinants from wild-type and mutant HEL*[a]

	11–25/A^d	23–32/A^d	71–85/A^d	106–116/E^d
W62R/HEL	1.1	1.3	1.1	0.3
W62K/HEL	1.0	1.2	1.2	0.3

[a] Dose-response curves from cocultures of LB27.4 APC with HEL variants were performed with T cell hybridomas specific for HEL determinants: 11–25/A^d (930B2); 23–32/A^d (6F7); 71–85/A^d (Ad71); and 106–116/E^d (G28.C9). Indicated is the ratio of EC_{50} (concentration of mutant or wild-type HEL required to achieve half-maximal stimulation) for each T cell hybridoma. Similar results were obtained in three independent experiments.

Discussion

In this study, we have demonstrated that the introduction of a dibasic motif adjacent to a subdominant determinant enhanced the presentation of this determinant from the whole molecule, as read out by its stimulatory capacity for specific T cell hybridomas.

According to the peptide controls, the introduced change does not affect TCR recognition of the peptide. Efficiency of fluid phase uptake of Ag by the B lymphomas is unlikely to be affected by the mutation and cannot account for the observed effect because the presentation of determinants other than 23–32/A^d were not equally enhanced by the F34R substitution (notably, equivalent presentation of the dominant determinant 106–116/E^d from wild-type HEL and F34R).

Because we are comparing the stimulatory capacity of two protein variants when presented by the same APC to the same T cell hybridoma, the only variable is the peptide mixture presented on the relevant MHC molecule ("determinant display"). There are several types of change in this peptide mixture that could account for the observed increase in stimulatory capacity: 1) quantitative, facilitating proteolytic cleavage near the determinant could simply allow for a more efficient generation of basically the same set of stimulatory peptide species; and 2) qualitative, the composition of the peptide mixture could be altered to contain peptides with higher stimulatory capacities or fewer peptides with antagonistic properties (15) without even altering the total amount of displayed peptides containing the core. Although the experiments described in this study do not discriminate between these possibilities (and the observed effect may well be mediated by a combination of both), our results strongly suggest that the increase in T cell hybridoma stimulation is a result of differential processing of HEL and F34R proteins by the APC.

What makes F34R a more efficient substrate for the generation of 23–32/A^d by APC? One report has suggested that the efficiency of presentation of the dominant determinant 106–116/E^d by APC, as well as the susceptibility to in vitro proteolytic cleavage, was inversely correlated to the stability of the chemical HEL variants (16).

Importantly, we find that presentation of the determinant 106–116/E^d from F34R is not enhanced (Fig. 3*A*). Furthermore, just like HEL, F34R was not susceptible to proteolytic cleavage in its native form, whereas reduced and S-3-(trimethylated amino) propylated HEL (srHEL) and srF34R were equally susceptible to cleavage by cathepsins D, L, and S and lysosomal fractions in vitro (C. Schroeter and H. Kalbacher, personal communication). These data argue strongly against F34R being generally more susceptible to proteolytic cleavage.

Although the current data do not exclude that the change at position 34 affects an unknown endopeptidase motif rather than facilitating cleavage at the dibasic site, we favor the conclusion that the introduced dibasic site is specifically recognized by an endopeptidase and that scission at this position allows more efficient generation of the determinant. This interpretation is supported by the finding that the mutant F34K enhances display to an equivalent degree to that of F34R (data not shown). Furthermore, the destruction of one of the two dibasic sites that naturally occur in the HEL sequence (by altering position K96K97 to K96L97) affects the presentation of the adjacent cryptic determinant 87–96/E^k (A.A., unpublished results). In addition, the introduction of a dibasic motif adjacent to a codominant determinant in the H-2^k haplotype, 46–61/A^k, also specifically affected the presentation of this determinant (H.D., unpublished results). Taken together, our results strongly suggest an involvement of proteolytic cleavage at dibasic motifs in processing of HEL for presentation on MHC class II. Importantly, the mutant proteins W62K and W62R had stimulatory capacity comparable to that of wild-type HEL for the determinants analyzed in the H-2^d haplotype, indicating that the localization of the dibasic motif is crucial for its effect on processing of specific determinants.

The eight members of the family of proprotein convertases known to date have diverse patterns of tissue distribution, intracellular localization, and recognition motifs (some requiring additional basic amino acids) (7, 8). We are currently investigating whether any of them, or a novel member, could be responsible for mediating the effect described in this report.

Factors affecting determinant display have important implications for immunodominance, i.e., the fact that the immune response toward complex pathogens as well as to immunization with experimental protein Ags is directed toward only a limited number of all theoretically possible determinants of the inciting Ags (17, 18). In some cases there is a direct relationship between the amount of a family of naturally processed peptides related to a given determinant that can be eluted from class II proteins and its position in the hierarchy of immunodominance (19). Some subdominant or cryptic determinants may even be displayed at levels so low as to make their quantitation by elution from MHC molecules extremely challenging (20). On the other hand, it is important to point out that even very well-displayed determinants can be functionally cryptic because of limitations in the T cell repertoire (17, 21, 22). Clearly, sufficient display of a determinant is a necessary but not sufficient requirement for an immune response directed toward it. This notion is underscored by a report demonstrating an Ag-processing threshold for in vivo T cell activation, whereas further enhancement of processing efficiency beyond this threshold did not further increase the in vivo T cell response (23).

These considerations are pivotal for vaccine approaches that rely on the insertion of defined determinants into carrier proteins (24–28). Knowledge of endopeptidase cleavage motifs will be necessary for the rational design of such vaccines. The approach described in this study can be applied to clarify the role of potential endopeptidase cleavage motifs for the generation and display of T cell determinants.

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