

CD8⁺ T Cell Recognition of an Endogenously Processed Epitope Is Regulated Primarily by Residues within the Epitope

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Summary

Cytotoxic T lymphocytes (CTL) recognize short antigenic peptides associated with cell surface class I major histocompatibility complex (MHC) molecules. This association presumably occurs between newly synthesized class I MHC molecules and peptide fragments in a pre-Golgi compartment. Little is known about the factors that regulate the formation of these antigenic peptide fragments within the cell. To examine the role of residues within a core epitope and in the flanking sequences for the generation and presentation of the newly synthesized peptide fragment recognized by CD8⁺ CTL, we have mutagenized the coding sequence for the CTL epitope spanning residues 202–221 in the influenza A/Japan/57 hemagglutinin (HA). In this study over 60 substitution mutations in the epitope were tested for their effects on target cell sensitization using a cytoplasmic viral expression system. The HA202–221 site contains two overlapping subsites defined by CTL clones 11-1 and 40-2. Mutations in HA residues 204–213 or residues 210–219 often abolished target cell lysis by CTL clones 11-1 and 40-2, respectively. Although residues outside the core epitope did not usually affect the ability to be lysed by CTL clones, substitution of a Gly residue for Val-214 abolished lysis by clone 11-1. These data suggest that residues within a site that affect MHC binding and T cell receptor recognition appear to play the predominant role in dictating the formation of the antigenic complex recognized by CD8⁺ CTL, and therefore the antigenicity of the protein antigen presented to CD8⁺ T cells. Most alterations in residues flanking the endogenously expressed epitope do not appreciably affect the generation and recognition of the site.

CD8⁺ CTL recognize nonnative fragments of proteins associated with MHC class I molecules (1). This association presumably occurs between newly synthesized MHC class I molecules and peptide fragments in a pre-Golgi compartment (2–4). Peptide fragments are generated as a result of protein fragmentation in the cytoplasm and possibly in the endoplasmic reticulum (ER).¹ Recent evidence suggests that the peptide fragments associated with MHC class I molecules are 8–10 amino acids in length (5–8) and that MHC-linked genes may encode products involved in the fragmentation of cytosolic proteins (9–13) and transport of peptide fragments from the cytoplasm to the ER. Although there is an increasing body of information on the structure of MHC-peptide complexes, little is known about the formation of these antigenic fragments within the cell and the role of residues within and surrounding an antigenic epitope in the

generation of the endogenously processed product, its transportation, and the formation of the complex between newly synthesized endogenous peptide fragments and MHC class I molecules (14, 15).

The influenza A/Japan/57 virus contains an immunodominant site that is recognized by CD8⁺ CTL in association with the H-2 K^d molecule. This site encompasses two distinct partially overlapping subsites recognized by different subsets of K^d-restricted CTL (16). These subsites span hemagglutinin (HA) residues 204–213 (LYQNVGTYVS) and 210–219 (TYVSVGTSTL) as defined by expression of minigenes encoding truncated portions of the 202–221 site (Hahn et al., manuscript in submitted for publication) and synthetic peptides (Myers et al., manuscript in preparation) (see Fig. 1 a). CTL clone 11-1 recognizes the HA204–213 site, whereas clone 40-2 recognizes the HA210–219 site.

To examine the importance of residues within the core epitope and flanking site in the processing and presentation of the newly synthesized peptide product, we have carried out

¹ Abbreviations used in this paper: ds, double-stranded; ER, endoplasmic reticulum; HA, hemagglutinin; NP, nucleoprotein.

saturation mutagenesis in a synthetic minigene encoding the 20-amino acid HA202–221 site and expressed the mutant constructs using a double subgenomic Sindbis virus (dsSIN) expression vector (17). Since the NH₂-terminal residues flanking the 210–219 site, i.e., residues 202–209, lie in part within the 204–213 site, we could simultaneously assess the effect of mutations within the NH₂-terminal site, i.e., at residue 204–209, on recognition of the COOH-terminal 210–219 site. Likewise, mutations within the COOH-terminal site would be assessed for their impact on the presentation of the NH₂-terminal site. We have found that mutations within the coding sequence of an endogenous epitope affect T cell recognition. With one exception alterations in flanking residues do not substantially affect the formation of the epitope recognized by CD8⁺ T cells. Thus, residues within a site that affect MHC binding and TCR recognition appear to play the predominant role in dictating the formation of the antigenic complex recognized by CD8⁺ CTL and therefore the antigenicity of the protein antigen for CD8⁺ T cells.

Materials and Methods

Saturation Mutagenesis. Two different parental HA202–221 constructs were utilized for saturation mutagenesis of the NH₂- and COOH-terminal epitopes. For mutants 1–31, the parental construct was a Met residue followed by HA residues 202–221 and 21 unrelated amino acids. For mutants 32–60, the parental construct began with a Met residue followed by HA residues 198–221 and the same 21 unrelated amino acids. Each of these parental constructs gave similar lysis values when tested with CTL clones 11-1 and 40-2. These minigenes were made by cloning appropriate synthetic double-stranded (ds) oligonucleotide adaptors with flanking 5' XbaI and 3' BamHI cohesive ends into pH3'2J1, a phagemid shuttle vector designed for constructing Sindbis virus recombinants (17). To facilitate saturation mutagenesis of either the NH₂-terminal (HA202–212) or the COOH-terminal epitope (HA211–221), silent changes were used to introduce a KpnI site at residues 209 and 210. For saturation mutagenesis, the XbaI-KpnI or the KpnI-BamHI segments were replaced by ds oligonucleotides containing a mixture of bases (1.7 or 2% contamination with the other three bases, respectively) at all positions. Separate oligonucleotides were used to mutate Gly-209. Pools of mutant plasmid DNAs were prepared (18), and the ApaI-XhoI restriction fragment containing the Sindbis virus subgenomic promoter, the HA minigene, and the Sindbis virus 3' terminus was used to replace the corresponding region of the full-length Sindbis vector cDNA clone (17). Individual clones were isolated, their sequences determined (18), and a subset was selected for target cell sensitization studies. Infectious recombinant Sindbis virus stocks of these mutants were rescued by *in vitro* transcription of infectious RNA and transfection of BHK-21 cells (19). Subgenomic RNA production has been analyzed for the negatives, or surprising mutants.

Cell Lines and CTL Clones. The P815 (H-2^d) mastocytoma cells were maintained in DME supplemented with 10% (vol/vol) FCS and 1% (wt/vol) glutamine. CTL clones 11-1 and 40-2 were isolated CTL precursors primed with influenza A/Japan/305/57 virus and established as previously described (20). They were passaged weekly in the presence of irradiated influenza A/Japan/305/57-infected splenocytes and a source of exogenous T cell growth factor.

Virus Stocks. Influenza A/Japan/305/57 virus (H2N2 subtype) was grown in the allantoic cavity of 10-d-old chicken embryo and stored as infectious allantoic fluid (21). dsSIN stocks were produced by lipofectin-mediated transfection of BHK-21 cells using 5' capped RNA derived by SP6 RNA polymerase transcription of XhoI-linearized plasmid templates (19). Culture supernatants were harvested at 24 h postinfection and aliquots stored at –80°C until use. dsSIN virus titers were determined by plaque assay on monolayers of chicken embryo fibroblasts.

CTL-mediated Cytolysis Assay. The P815 (H-2^d) cells were used as target cells in a ⁵¹Cr-release assay. 10⁶ target cells were infected for 1 h at 37°C with the various dsSIN recombinants (at 50 PFU/cell) and labeled with 150 μCi ⁵¹Cr for 2 h at 37°C. After washing to remove free label, target cells were incubated with CTL clones for 6 h at 37°C as described previously (20). The E/T ratio was 5:1. The percentage of specific ⁵¹Cr release was determined by standard procedures (22). Values for ⁵¹Cr release were the mean of quadruplicate samples and SDs were typically <5%.

Results and Discussion

Generation of Mutants in the HA202–221 Site by Saturation Mutagenesis. The HA 202–221 site recognized by CD8⁺ CTL in association with the H-2 K^d molecule has been mapped to two overlapping subsites spanning HA residues 204–213 (LYQNVGTYVS) and 210–219 (TYVSVGTSTL) by analyzing minigenes encoding truncated portions of the 202–221 site. To investigate the role of individual amino acid residues within the core epitope, as well as the flanking residues, we have performed the saturation mutagenesis in two overlapping CTL epitopes using ds oligonucleotides containing a mixture of bases at all positions. Our strategy was to express 42–46 amino acid-long peptides. The rationale was to ensure a product of adequate length for effective proteolytic processing and to avoid any interference due to variability in length of the minigene product.

These mutations were rescued using infectious dsSIN recombinant. We have generated >70 mutants containing single as well as multiple substitutions. P815 target cells expressing the mutant epitopes were tested for recognition by two representative K^d-restricted CD8⁺ CTL clones directed to HA202–221. One clone, 11-1, is directed to the NH₂-terminal 204–213 site, while the other clone, 40-2, is directed to the COOH-terminal 210–219 site.

Recognition of COOH-terminal Epitope Mutants by Clone 40-2. Mutations in the flanking residues 202–209 had little effect on the recognition of the COOH-terminal 210–219 site by clone 40-2 (Fig. 2). The first substitution to affect T cell recognition was at position 210, where the Thr→Asn change resulted in diminished recognition of the COOH-terminal 210–219 site. A Thr→Gly substitution at this position did not affect lysis by 40-2. Mutations in residues 211–219 in most cases profoundly affected recognition of the product by clone 40-2, while alterations in the COOH-terminal flanking residues 220 Asn and 221 Lys had no effect on recognition. Taken together, these data suggest that mutations in residues outside the minimal endogenous 210–219 epitope have little effect on presentation and recognition of this endogenous product.

Recognition by clone 11-1

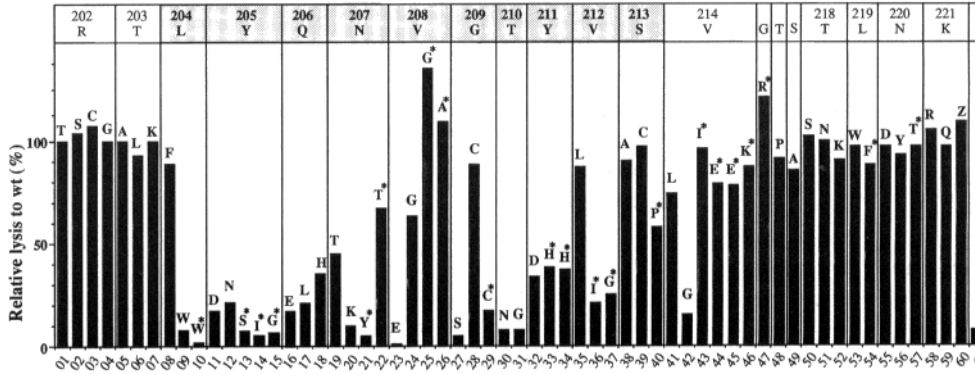


Figure 3. Recognition of mutant HA peptides by the NH₂-terminal epitope-specific clone 11-1. Percent of relative lysis to wild type was calculated as in Fig. 2. Assays were repeated at least three times and the relative values were consistent. The level of lysis for parental construct was 50% for clone 11-1 and nonspecific lysis for the negative control, SIN:027, was 10% for CTL clone 11-1.

ognition of the endogenous product by clone 11-1. On the other hand, mutations in residues 204–213, which define the minimal endogenous epitope, usually resulted in diminished or abolished lysis by clone 11-1.

The NH₂-terminal epitope (LYQNVGTYVS) contains a K^d-restricted peptide motif similar to that identified by sequence analysis of the HPLC-purified peptide population bound to K^d molecules (7). In these studies, the majority of K^d-bound peptides appeared to be nonapeptides and contained a nonrandom amino acid composition at each position. For the second position, the predominant residue identified was Tyr with significant levels of Phe, which has a side chain similar to that of Tyr, and at position 9, the residues preferred were Ile and Leu. At all other positions a larger, but distinct, subset of residues was found. A comparison of this motif with previously defined K^d-restricted epitopes (7) indicated that, if aligned by the Tyr residue, most fit quite well with the consensus K^d-restricted nonamer motif, indicating that preferred residues, such as Tyr position 2 and Leu or Ile at position 9, may represent critical anchor residues for peptide-MHC interaction. The NH₂-terminal epitope fits this motif with Tyr-205 and Val-212 acting as anchors at positions 2 and 9. Parallel studies with synthetic peptides suggest that the Tyr-205 and Val-212 are critical for peptide binding to K^d. However, in this site other residues, with the exception of Asn 207 and Val 208, also appear to affect peptide binding for K^d (Myers et al., manuscript in preparation) and therefore recognition by clone 11-1. Substitution of Tyr-205 (the proposed anchor residue) with Asp or Asn abolished lysis completely. Substitution of Val-212 (the proposed anchor residue) by Ile or Gly abolished the lysis by CTL clone 11-1, whereas substitution with Leu allowed recognition by 11-1. These results are interesting since the isolated endogenous K^d-restricted peptides appear to predominantly utilize Ile and Leu to anchor the residues to K^d molecules (7). This suggests that Val-212 may interact with the clone 11-1 TCR as well as anchor the peptide to MHC molecules.

Replacement of Leu-204 by Phe is tolerated but results in a decreased lysis by CTL clone 11-1. Replacement with a bulkier Trp residue abolished lysis, indicating that size of the side chain may be important for peptide interaction with MHC or TCR. Asn-207 has been proposed to interact with the

TCR based on sequence comparison of different influenza virus strains (16), and Asn-207 could be replaced with Thr but not with Lys. Val-208 could be replaced with by either Ala or Gly but not with Glu, suggesting that either the size or the hydrophobicity of the side chain could be important at this position. Substitution of Gly-209 with Cys but not with Ser was tolerated but led to less efficient lysis. This is rather surprising given the structural similarity of Ser and Cys, and suggests that stringent size constraints may exist at this position. Studies with synthetic peptides suggests that Gly-209 may contribute both to MHC interaction and TCR contact. Ser-213 could be replaced with Ala and Cys but substitution with Pro resulted in a decreased lysis by clone 11-1.

As observed for the residues flanking the 210–219 site, most mutations in the residues flanking the 11-1 site at its COOH terminus, i.e., residues 214–221 like the mutations in the NH₂-terminal 202 and 203 positions, had no effect on recognition of the site by clone 40-2. A notable exception was the minigene with the Gly change at position 214 (construct 42), which was not lysed by clone 11-1. Other substitutions (e.g., Val→Leu, Ile, Glu, Lys) at this position were tolerated (Fig. 3).

The Val→Gly mutation at position 214 was the only alteration outside a minimal site of >50 mutants examined to date that resulted in nearly complete loss of recognition of the endogenous product. One explanation for this finding is that the substitution of Gly at this position inhibits a processing/presentation event necessary for the formation of the peptide/K^d complex. Such an effect of flanking residues has been proposed from studies on CD8⁺ T cell recognition of a murine cytomegalovirus early gene product (14). It is interesting that Gly substitution at 214 led to the minimal endogenous peptide followed by two Gly residues at its COOH terminus. Double Gly residues at the COOH-terminal border of an epitope might play a negative role for antigen processing. This hypothesis is currently being examined.

In this report we have examined the role of flanking residues in the generation of the endogenous antigenic moiety recognized by CD8⁺ CTL with the aim of analyzing the impact of the surrounding amino acid residues on the immunogenicity of a site recognized by CD8⁺ CTL. Mutations in the HA204–213 region as expected affected the recognition by

clone 11-1, which recognizes an epitope encompassing these residues, while residues in the NH₂- or COOH-terminal flanking residues, with one notable exception, had no effect. Similarly, only residues within HA210–219 were important for lysis by clone 40-2. At face value, our data suggest that flanking residues per se may not play a determining role in dictating the immunogenicity of a site within a protein. Except for the 214 Val→Gly change in each instance where a mutation affected T cell recognition of the 202–221 minigene product, the mutation was in a residue within the putative minimal endogenous epitope. On the other hand, the Val→Gly mutation did not affect the generation of the 204–213 site per se since another CD8⁺ clone 14-1 also directed to 204–213 could recognize the gene product containing the Val→Gly mutation, albeit with a lower efficiency than the wild type (Y. S. Hahn, unpublished data). Therefore, the Val→Gly alteration at position 214 did result in the formation of an epitope recognizable by CD8⁺ T cells. Even though clone 11-1 can recognize minigenes encoding only residues 204–213 or 204–212 (albeit with a lower efficiency) (Hahn et al., manuscript submitted for publication), we can not exclude the intriguing possibility that the actual endogenous site recognized by clones 11-1 and 14-1 includes residue 214, and therefore that clone 11-1 but not clone 14-1 is sensitive to Val→Gly substitution at this position. Alternatively, substitution of the Gly at position 214 may decrease the efficiency of trimming of the endogenous product so that an 11-amino acid peptide containing 214 Gly is associated with the K^d cleft. This extended peptide would still be recognizable by clone 14-1 but not by clone 11-1. These and other alternatives, e.g., effect of Val→Gly substitution on peptide transport from the cytoplasm to ER, are currently being examined.

These data support our earlier work, which showed that reposition of the HA202–221 site in different positions along the full-length HA polypeptide did not detectably influence presentation of the antigenic moiety to MHC class I-restricted T cells (15). In the present report we have focused directly on the role of flanking sequences outside the antigenic site since in our earlier work two extra NH₂-terminal and COOH-terminal residues, i.e., Arg-202, Thr-203, Asn-220, and Lys-221, were also included with the repositioned minimal endogenous peptide. Our data are consistent with the study by Chimini et al. (24), which stated that CTL could recognize target cells transfected with recombinant genes encoding the 13-mer MHC class I-restricted T cell epitope inserted into the influenza virus nucleoprotein (NP) gene. Thus, alteration of the flanking regions did not prevent antigen processing and presentation. However, our results are in apparent contrast to two other studies in which residues outside of the antigenic site could alter processing and presentation of antigen to class I-restricted T cells. One was the observation by Del Val et al. (14) that an antigenic determinant from a murine cytomegalovirus protein was not efficiently presented when placed into certain regions of a heterologous protein.

Hence, flanking residues can play a critical role in determining the efficiency of antigen processing and presentation to the L^d-restricted CTL. Flanking Gly residues and negatively charged residues such as Asp were implicated as playing negative roles for efficient antigen presentation. The other observation was the report by Eisenlohr et al. (25) that the addition of two COOH-terminal residues corresponding to NP residues Thr-157 and Gly-158 to a minigene encoding a naturally processed determinant from influenza virus NP spanning amino acids 147–155 (TYQRTRALV) severely reduced presentation of the endogenously produced peptide to K^d-restricted CTL, whereas the minigene expressing residues 147–158 can efficiently sensitize target cells. However, its presentation was greatly enhanced by further extending to include all of the additional residues of NP (NP, Arg⁻). These data suggest that Thr and Gly residues can negatively affect the immunogenicity of minimal determinants NP147–155 but not the presentation of NP147–156 minigene product.

Several testable alternatives may account for these apparently conflicting observations on the effects of residues flanking core epitopes on recognition by CTL. A trivial possibility is that, with the exception of the Gly substitution at Val-214, the mutations examined in our study may not have included those that hinder proteolytic processing and/or peptide transport. Alternatively, the larger size of our minigene construct (42–46 residues) may allow efficient proteolytic processing and behave more like the full-length HA protein, especially since the study of Eisenlohr et al. (25) suggests that the negative effects of flanking sequences on the recognition of their 10–32-residue minigene products can be reversed by including all of the additional residues in NP. Another possibility is that the expression levels of minigene products by the Sindbis system may be too high to detect subtle differences in the efficiency of presentation and CTL recognition. Finally, it may be relevant that CTL clones used for this study have high-affinity TCRs and lyse target cells very efficiently, even at ratios of E/T cells as low as 1:1. Although it is difficult to correlate the level of specific lysis by CTL with different antigenic moieties, the level of specific lysis of target cells expressing the HA minigene in our studies using an E/T ratio of 5:1 is roughly equivalent to the recognition of CMV pp89 protein by specific CTL using an E/T ratio of 100:1 (14).

It is not yet clear that whether the processing of 42–46-amino acid long peptides that we have tested in this study occurs in the cytoplasm or whether they can be transported to the ER without any processing and further trimming occurs after formation of peptide-MHC complexes. In any case, future biochemical and cell biological investigations using defined CTL epitopes with mutations blocking functional presentation (like the Gly-214 substitution) should help to delineate discrete steps in the pathway as well as define the structural determinants of the antigens that affect proteolysis, transport, MHC association, and TCR recognition.

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